

# **INFLAMMATORY MEDIATORS IN HUMAN ENDOMETRIUM**

**Rebecca Lee Jones**

**PhD**

**The University of Edinburgh**

**1998**



## ABSTRACT

Implantation and menstruation share many features consistent with an inflammatory response. An accumulation of leukocytes in the endometrial stroma, accompanied by increased vascular permeability, is apparent in the mid-late secretory phase. Stromal leukocytes comprise macrophages and the uterine specific natural killer (NK)-like large granular lymphocytes (LGLs), which are believed to be essential for successful implantation. In the absence of pregnancy, an influx of neutrophils is observed in the immediate premenstrual phase. It is not known whether migration from the peripheral circulation contributes to the increase in numbers. A typical C-X-C chemokine, interleukin-8 (IL-8), selectively attracts and activates neutrophils. In contrast, a C-C chemokine, monocyte chemoattractant protein-1 (MCP-1) stimulates the chemotaxis and activation of monocytes, NK cells and T lymphocytes. Elevated concentrations of prostaglandins  $E_2$  and  $F_{2\alpha}$  ( $PGE_2$  and  $PGF_{2\alpha}$ ), potent modulators of vascular permeability, are detectable in the perimenstrual phase. Prostaglandins (PGs) are synthesised by cyclooxygenase (COX), of which two isoforms exist, the constitutively expressed COX-1 and inducible COX-2.

The present study has investigated the expression of the chemokines IL-8 and MCP-1, and COX-2 in the human non-pregnant and pregnant endometrium. Both IL-8 and MCP-1 were localised by immunohistochemistry to the perivascular cells of all blood vessel types in the endometrium. Similarly COX-2 immunostaining was present in the vasculature and glandular epithelium. Reduced immunoreactivity was detectable in the periovulatory, early and mid secretory phases. Maximal levels were identified in the premenstrual or late secretory phase, coinciding with progesterone withdrawal and in addition, with the accumulation of leukocytes in uterine stroma. In first trimester decidua, low levels of expression were observed for IL-8 and COX-2, with slightly higher levels for MCP-1. This pattern was confirmed by measurement of mRNA levels by non-competitive semi-quantitative RT-PCR. Equal loading of RNA samples was ensured by measurement of GAP-DH expression levels. RT-PCR product yield for GAP-DH, IL-8, MCP-1 and COX-2 was determined by ELISA. Maximal expression was observed in menstrual phase samples. Decidual mRNA expression patterns were consistent with immunohistochemical data.

The role of progesterone in regulation of local mediator expression was examined



by immunohistochemical analysis of endometrium and decidua obtained from women with clinically perturbed progesterone concentrations. Additionally, RNA extracted from tissue from timed endometrial biopsies was subjected to RT-PCR for GAP-DH, IL-8, MCP-1 and COX-2. 48 hours after exogenous progesterone withdrawal a significant elevation in IL-8 and COX-2 mRNA expression was detected. Further supportive evidence for an inhibitory role for progesterone was the low expression of IL-8 and COX-2 mRNA in women administered hCG to extend the lifespan of the corpus luteum. Elevated endometrial immunoexpression of IL-8 and COX-2 was detected in the first few months after exposure to intra-uterine levonorgestrel, declining with long term exposure. An *in vitro* culture system was employed to investigate further chemokine and COX-2 expression. Endometrial explants were cultured for 2 hours with serum, phorbol myristate acetate (PMA) and progesterone. RNA extracted was subjected to amplification by RT-PCR for GAP-DH, IL-8, MCP-1 and COX-2. A dramatic elevation in the number of mRNA transcripts for IL-8, MCP-1 and COX-2 was detected following culture with serum, which was augmented by the addition of PMA. No significant decrease in expression was observed in the presence of progesterone.

A further aspect of this study was to examine a putative immunomodulatory role of endometrial prolactin (PRL). PRL is produced by decidualised stromal cells and is believed to function as an autocrine / paracrine factor in the uterus. The localisation and temporal expression of PRL-R in the endometrium was investigated. Strong immunoreactivity was displayed by a subset of stromal cells and glandular epithelium, from the mid-late secretory phase and continuing into early pregnancy. This is indicative of a role in decidualisation and paracrine regulation of glandular activity. The temporal and spatial expression of the chemokines is in agreement with a role in facilitating leukocyte recruitment to the endometrium. This further supports both the involvement of IL-8, MCP-1 and PGs in the initiation of menstruation, and a downregulation by progesterone. PRL-R are present in the endometrium at a time when leukocyte proliferation / differentiation occurs, reinforcing an immunomodulatory role for PRL in the uterus.

## TABLE OF CONTENTS

<b>Abstract</b>	2
<b>Declaration</b>	7
<b>Acknowledgements</b>	8
<b>Abbreviations</b>	9
<b>Chapter 1      General Introduction and Literature Review</b>	<b>12</b>
1.1      Human Endometrium and Menstrual Cycle	13
1.11      Proliferative Phase	13
1.12      Ovulation and Secretory Phase	13
1.13      Menstruation	16
1.14      Implantation	19
1.2      Sex Steroid Hormones	23
1.3      Endometrial Leukocytes	26
1.4      Leukocyte Migration	31
1.5      Inflammatory Mediators	33
1.51      Chemokines	33
1.52      Chemokine Receptors	38
1.53      Reproductive Significance of Chemokines	39
1.54      Cytokines	42
1.55      Nitric Oxide	45
1.56      Prostaglandins	46
1.6      Prolactin	50
1.7      Hypothesis	52
1.8      Aims	55
<b>Chapter 2      General Materials and Methods</b>	<b>56</b>
2.1      Materials	57
2.2      Methods	62
2.21      Tissue Collection and Patient Details	62
2.22      Immunohistochemistry	64
2.23      Scoring and Analysis of Immunostaining	67
2.24      RNA Extraction from Whole Tissue	68
2.25      Non-competitive Semi-quantitative RT-PCR	68
2.26      ELISA	80

<b>Chapter 3</b>	<b>Localisation and Temporal Expression of Chemokines and Cyclooxygenase-2 in Human Endometrium</b>	94
3.1	Introduction	95
3.2	Methods	97
3.3	Results	99
3.4	Discussion	127
<b>Chapter 4</b>	<b><i>In Vivo</i> perturbation of Local Progestogen Environment: Effect on endometrial Chemokine and COX-2 Expression</b>	131
4.1	Introduction	132
4.2	Methods	135
4.3	Results	139
4.4	Discussion	166
<b>Chapter 5</b>	<b>Examination of Inflammatory Mediator Expression using an <i>in vitro</i> Culture System</b>	171
5.1	Introduction	172
5.2	Methods	179
5.3	Results	181
5.4	Discussion	211
<b>Chapter 6</b>	<b>Localisation and Temporal Expression of Prolactin Receptor in Human Endometrium</b>	215
6.1	Introduction	216
6.2	Methods	218
6.3	Results	221
6.4	Discussion	235
<b>Chapter 7</b>	<b>General Discussion</b>	240
7.1	Expression of inflammatory mediators in human endometrium	241
7.2	Roles of locally acting mediators in preparation of the endometrium for implantation	245
7.3	Involvement of inflammatory mediators in the initiation of menstruation	248
7.4	Future Studies	250

<b>Bibliography</b>		252
<b>Appendices</b>	I	Recipes for Solutions
	II	RT-PCR Protocols
	III	Conference Presentations
	IV	Publications
		303
		306
		308
		310

## DECLARATION

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Rebecca Jones

## ACKNOWLEDGEMENTS

I would like to thank Dr Hilary Critchley for her constant supervision, help and advice throughout the course of my PhD, especially during the writing of this thesis. Similarly, I wish to thank Dr Rodney Kelly for his enthusiastic supervision and help with the molecular biology studies. Many thanks to Miss Teresa Drudy for her technical expertise and constant support and encouragement. Thanks are also due to Dr Stuart Milne, Dr Hong Wang, Mrs Sylvia Pinder, Miss Gail Baldie, Dr Fiona Denison and Miss Vivien Grant for making the lab such a pleasant place to work.

I acknowledge the help of Dr Faye Roger, Miss Pawlina Lague, Miss Sheila Boddy and Dr Stuart Milne for the sequencing of RT-PCR products, and to Miss Joanne McVerry for assistance with Southern Hybridisation.

I extend my thanks to Professor Alan McNeilly, Dr Julie Brooks and Dr Henry Jabbour for their kind supervision of the prolactin receptor studies, and to Professor David Baird for his help with clinical studies. Likewise, I would like to thank all the clinicians and theatre staff at the Royal Infirmary of Edinburgh, especially Dr Hilary Critchley, Dr Andrew Paterson and Sister Catherine Hall, who assisted in the collection of endometrial samples.

I am very grateful to Mrs Vicky Watters, Miss Morag Burns and Mrs Carol Irvine for their assistance and advice with secretarial matters, and to Mr Tom McFetters, Mr Ted Pinner and Mr Mike Millar for help with all aspects of photography and graphical illustrations.

Finally, I would like to thank everyone in the Centre for Reproductive Biology, my family and friends for their support and encouragement during the past three years.

## ABBREVIATIONS

ABC-HRP	Avidin-Biotin Complex, conjugated to horseradish peroxidase
AMV	Avian Myeloblastosis Virus
BSA	Bovine Serum Albumin
COX-2	Cyclooxygenase-2
CAMs	Cell Adhesion Molecules
CD	Cell Differentiation antigen
DAB	3, 3' - Diaminobenzidine
DEPC	Diethyl pyrocarbonate
E <sub>2</sub>	Oestradiol
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunoabsorbant Assay
ER	Oestrogen Receptor
FCS	Fetal Calf Serum
FSH	Follicle Stimulating Hormone
GAP-DH	Glyceraldehyde Phosphate - Dehydrogenase
hCG	Human Chorionic Gonadotrophin
HLA	Human Leukocyte Antigen
IL-8	Interleukin-8
IL-1	Interleukin-1
IgG	Immunoglobulin G
Jak	Janus Kinase
LGL	Large Granular Lymphocytes
LH	Luteinising Hormone

LIF	Leukaemia Inhibitory Factor
Lng-IUS	Levonorgestrel-releasing Intra Uterine System
MCP-1	Monocyte Chemoattractant Protein-1
MCP-2, -3, -4, -5	Monocyte Chemoattractant Protein-2, -3, -4, -5
MIP-1 $\alpha$ , -1 $\beta$	Macrophage Inflammatory Protein-1 $\alpha$ , -1 $\beta$
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
NBF	Neutral Buffered Formalin
NK	Natural Killer cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGs	Prostaglandins
PGD <sub>2</sub>	Prostaglandin D <sub>2</sub>
PGDH	Prostaglandin Dehydrogenase
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
PGF <sub>2a</sub>	Prostaglandin F <sub>2</sub>
PGHS	Prostaglandin H Endoperoxide Synthase (synonym: Cyclooxygenase)
PGI <sub>2</sub>	Prostaglandin I <sub>2</sub>
PgR	Progesterone Receptor
PMA	Phorbol 12-Myristate 13-Acetate
PRL	Prolactin
PRL-R	Prolactin Receptor
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RNA	Ribonucleic Acid
RT	Reverse Transcriptase



RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RU486	Mifepristone
Stat	Signal Transducer and Activator of Transcription
TBS	Tris Buffered Saline
TGF	Transforming Growth Factor
Th1	T Helper cell type 1
Th2	T Helper cell type 2
TIMPS	Tissue Inhibitors of Matrix Metalloproteinases
TNF- $\alpha$	Tumour Necrosis Factor-Alpha
TXA <sub>2</sub>	Thromboxane A <sub>2</sub>
VEGF	Vascular Endothelial Growth Factor

## **CHAPTER 1**

### **GENERAL INTRODUCTION & LITERATURE REVIEW**

## **1.1 The Human Endometrium and Menstrual Cycle**

The human endometrium undergoes cyclical activity, with well defined phases of proliferation, differentiation and degradation (Carson, 1992). This provides a unique and specialised environment for the implantation of a blastocyst and modulates subsequent invasion of trophoblast and placentation. In the absence of pregnancy, the functional layer of the endometrium is shed during menses and rapidly replaced during the following proliferative phase. These changes are governed by the endocrine sex steroid hormones oestradiol and progesterone (Johannisson et al., 1982), released from the ovarian follicle and corpus luteum respectively under the control of the pituitary gonadotrophin hormones follicle stimulating hormone (FSH) and luteinising hormone (LH). The histological and morphological features of the endometrium throughout the menstrual cycle have been described in detail (Noyes et al., 1950; Buckley et al., 1989; Strauss III et al., 1991; Yen, 1991).

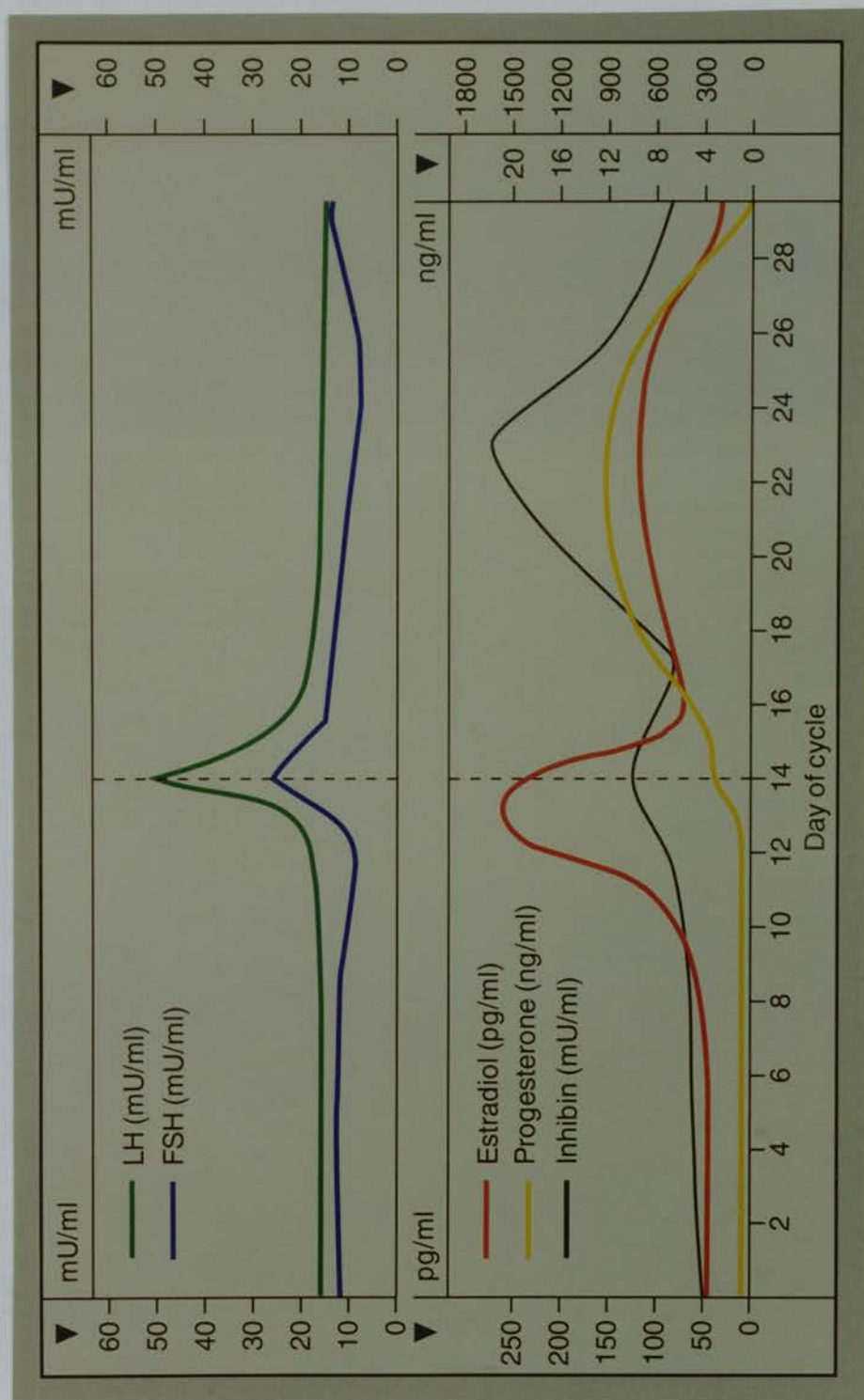
### **1.11 Proliferative Phase**

In the proliferative or follicular phase of the cycle, the upper layer of the endometrium (functionalis) undergoes rapid regeneration from the basalis endometrium which is retained after menstruation (Haines et al., 1987). Progressively increasing circulatory levels of oestradiol from the maturing follicle direct extensive proliferation of stromal, glandular epithelial cells and vascular elements. Regeneration is completed by day 5 from the last menstrual period (LMP), producing a tightly packed stroma of fibroblast-like cells and straight, narrow, tubular glands comprised of columnar epithelial cells with a low nucleus, interspersed with small vessels and capillary networks. Mitotic activity is apparent throughout the proliferative phase, extending three days post ovulation. The proliferative phase is variable in length and may be subdivided into the early (day 5 - 7), mid (day 8 - 10) and late (day 11 - 14) proliferative stages, each displaying subtle differences in histological appearance. The late proliferative stage, is especially characterised by extensive glandular growth and pseudo-stratification of the epithelial cells.

### **1.12 Ovulation and Secretory Phase**

Ovulation is triggered by the LH surge (around day 14) which in turn is stimulated by a peak in oestradiol levels. Luteinisation of the corpus luteum results in production of progesterone and oestradiol from the steroidogenic luteal cells.

Progesterone is essential for the initiation and maintenance of pregnancy, which is reflected by its continuous production throughout the gestational period, initially from the corpus luteum until the placenta takes over steroid production (Csapo et al., 1973). Many endometrial histological changes are associated with the progesterone dominated secretory or luteal phase. The luteal phase is generally of constant length (14 days) and obvious histological differences are identifiable between early (days 15 - 18), mid (days 19 - 24) and late (days 25 - menses) secretory phases. One of the first indications that ovulation has taken place is the appearance of subnuclear intracytoplasmic glycogen rich vacuoles in the glandular epithelial cells. This causes displacement of the nuclei and the glands become tortuous and increase in diameter. By the mid secretory phase (days 18 - 24), acidophilic secretions are evident in the glandular lumen, comprising glycoproteins, uteroglobulin-like protein, pregnancy-associated endometrial protein (PEP) and pregnancy-associated endometrial  $\alpha_2$  globulin ( $\alpha_2$ PEG) (synonyms: placental protein 14 (PP14), endometrial protein 15, progesterone-dependent endometrial protein). Although the precise physiological functions of these secretions are unclear, the temporal production indicates a role in the preparation of the uterus for implantation, with visible secretory activity peaking at day 20 - 21. Stromal changes are also evident, with increased vascularisation accompanied by fluid filled oedema. The first predecidual transformations are detectable from day 23, with the development of spiral arterioles. Under progesterone control, closely associated stromal cells are seen to enlarge and become rounded, with the acquisition of eosinophilic cytoplasm and become surrounded by a thick pericellular matrix. In the late secretory or premenstrual phase cessation of glandular secretory activity ensues and glands become dilated and increasingly tortuous ("sawtoothed appearance"). An accumulation of bone marrow derived cells is apparent in the stroma. These morphological changes characterising decidualisation (Finn et al., 1984) "spread" throughout the functional endometrium and proceed into deeper layers if pregnancy occurs (Bell, 1991). First trimester decidua possesses all the above described characteristics of the pseudo-decidualised endometrium.



**Figure 1** Fluctuation of sex hormone concentrations during the menstrual cycle.

### 1.13 Menstruation

In the absence of implantation and pregnancy, the terminally differentiated decidualised endometrium must be shed to allow regrowth of the endometrium. Failure of a human chorionic gonadotrophin (hCG) signal production from an implanted blastocyst within 7 days of ovulation results in luteolysis and regression of the corpus luteum (Yen, 1991). Thus, production of progesterone and oestradiol ceases, resulting in the breakdown of the endometrium functionalis. Withdrawal of the steroids affects the integrity of the spiral arterioles and eventually leads to bleeding. This process facilitates the shedding of the endometrium and menses.

The shedding of the endometrium at the end of each cycle is a phenomenon almost unique to humans, the exceptions being old world primates, elephant shrews (*Elephantulus myurus jamesoni*) and certain species of bats (*Glossophaga soricina* and *Carollia*). Although the evolution of this wasteful process is not understood, it is believed to be a reaction to the extensive invasion that characterises the implantation of the human trophoblast (Finn, 1986; Finn, 1994). To regulate such a potentially dangerous event, maternal endometrium responds by becoming terminally differentiated. During the luteal phase, stromal cells undergo decidualisation, an accumulation of leukocytes in the stroma is apparent and extensive vascularisation occurs, most notably with the formation of the specialised spiral arterioles. These changes in stroma and the presence of bone marrow derived cells enables the production of a variety of local factors capable of modulating invasion. Decidual cell formation and maintenance and the integrity of the vasculature are entirely dependent on the presence of progesterone (Finn, 1986). When the steroid hormones are withdrawn in a non-conception cycle, the decidualised endometrium is sloughed off to allow the regrowth of the endometrium functionalis. Other animals, such as mice, also experience invasive implantation and the endometrium undergoes profound cellular and vascular changes in line with the above outlined theory. However, this decidualisation only occurs when stimulated by the presence of a blastocyst in the uterine lumen, unlike in women where decidualisation occurs in every menstrual cycle, and does not culminate in menses. Menstruation may be simulated in ovariectomised mice by the induction of pseudodecidualisation by placing a drop of oil in the steroid primed endometrium. Withdrawal of steroid treatment results in the initiation of menses, with an accompanying leukocyte infiltrate (Finn et al., 1984).



Little is known about the cellular interactions occurring during normal or dysfunctional menstruation. Pioneering investigation into the morphological events involved in menstruation were conducted in 1940 by Markee (Markee, 1940). Vascular and cellular changes in endometrium transplanted in the anterior chamber of the eye of the *rhesus macaque* were observed during the perimenstrual and menstrual stages. The first sign of impending menses was the apparent slowing of blood in the functionalis vasculature. This was accompanied by a dramatic reduction in thickness of the endometrium, presumably due to the reabsorption of the stromal oedema. Consequently, the highly coiled spiral arterioles were observed to become compressed, attributed to the "shrinkage" of the tissue, and intense vasoconstriction of spiral arterioles occurs. This induced a hypoxic state, leading to degradation of vessel walls and bleeding from focal points. Fragmented tissue was eliminated by blood flow from damaged vessels during menses which lasts 3 - 5 days. With the cessation of bleeding, regeneration of vasculature and endometrial stromal and epithelium occurred directed by oestradiol.

The molecular events and biochemical signalling involved in these processes is still unknown. Recent ultrastructural investigations (Ludwig et al., 1991; Roberts et al., 1992) have revealed lesions at focal points in both epithelium and stroma and a significant breakdown of the extracellular matrix (ECM), forming both the basal lamina of endothelial and epithelial cells and the interstitial matrix. Considerable interest has consequently been directed into the role of matrix metalloproteinases (MMPs) in this degradation. This family of secreted zinc proteins comprises the collagenases, gelatinases and stromelysins, each possessing preferential degradative activity to particular substrates. MMP-1 (interstitial collagenase) degrades collagen types I - III, VII and X; MMP-2 (gelatinase-A) degrades gelatins and collagen types IV, V, VII, XI; MMP-3 (stromelysin) degrades proteoglycans, fibronectin and laminin and MMP-9 (gelatinase-B) degrades gelatins and collagen types IV and V. MMPs-1, -2, -3 and -9 have all been detected in the human endometrium (Salamonsen, 1996a) and in combination would be capable of causing the degradation of all components of endometrial ECM. Regulation of MMP activity occurs at the translational level, with induction by a number of growth factors (epidermal growth factor (EGF), transforming growth factors- $\alpha$  and - $\beta$  (TGF- $\alpha$ , TGF- $\beta$ )) (Matrisian et al., 1990), cytokines (interleukin-1 (IL-1), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ )) (Salamonsen et al., 1996b), steroid hormones and oncogenes, and additionally post-translationally with the proteolytic activation of pro-enzymes. A further element of control is exerted through specific production of tissue inhibitors

of MMPs (TIMPS) (Nagase, 1991). mRNA transcripts for proMMP-1 and -3 are detectable in the endometrium in the period immediately prior to and during menses (Hampton et al., 1994), with elevations in the expression or activation of MMP-7, -2 and -9 in the premenstrual period (Salamonsen, 1996a). Furthermore, immunohistochemical localisation has defined a close spatial relationship between MMPs and regions of endometrial degradation (Brenner et al., 1996; Salamonsen et al., 1996b). The temporal expression is indicative of induction of transcription by progesterone withdrawal, and this has been verified by *in vitro* research (Marbaix et al., 1992; Marbaix et al., 1995; Brenner et al., 1996; Salamonsen et al., 1997). Moreover, an indirect regulation by the steroid via a number of endometrial lymphoid derived locally acting cytokines and proteases has been postulated for regulating MMP expression (Salamonsen et al., 1996b). Production of MMPs by specific endometrial leukocyte subtypes reinforces the role of migratory cells in the regulation of endometrial function (Shi et al., 1995; Vincent et al., 1997). Further investigations are being undertaken to verify the involvement and regulation of MMPs in the initiation of menstruation and additionally in the remodelling that occurs in the rapid regeneration of the proliferative endometrium. Recent publications have highlighted an additional role for MMPs-2 and -9 in the tissue remodelling associated with trophoblast invasion of the maternal decidua during implantation and placentation (Sharkey et al., 1996; Bjorn et al., 1997).

Since one of the first signs of impending menses is the intense vasoconstriction of the spiral arterioles, many investigations have attempted to isolate the vasoactive substances mediating this effect. Prostaglandins, locally acting mediators which have potent actions on vascular tone, have been implicated in the processes of menstruation, particularly as high concentrations are detectable in menstrual blood (Pickles et al., 1965). Prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) stimulates constriction of blood vessels and thus it was presumed that  $PGF_{2\alpha}$  was responsible for the vasoconstriction identified preceding menses. Recently, however, more attention has focused on a family of vasoconstrictor peptides called the endothelins, comprising endothelin-1 (ET-1), ET-2 and ET-3. Endothelin-1 is the most potent known vasoconstrictor, and has been detected in the human non-pregnant and pregnant endometrium localised to the glandular epithelium, with documented peaks in expression in the perimenstrual period (Cameron et al., 1992; O'Reilly et al., 1992; Cameron et al., 1993; Maggi et al., 1994; Ohbuchi et al., 1995). Furthermore, the enzyme responsible for the degradation of endothelin, neutral endopeptidase (NEP), is produced by the mid secretory phase endometrium, with markedly reduced



concentrations detectable premenstrually (Casey et al., 1991). An opposite situation, that is reduced endothelin and elevated NEP, appears to be occurring in the endometrium of women reporting menorrhagia (Marsh et al, 1997), or abnormal bleeding which is a common side effect experienced by users of progestin-only contraceptives (Marsh et al, 1995). Receptors for endothelin, (ET<sub>A</sub> and ET<sub>B</sub>) have been identified on a number of endometrial elements, including the endothelial cells of vessels, with alterations in subtype expression throughout the menstrual cycle (O'Reilly et al., 1992). Most notably, ET<sub>B</sub> is selectively expressed by the decidualised stromal cells, both during first trimester decidua and in endometrium exposed to synthetic progestin via an intra-uterine device (Kohnen et al., 1997). Different functions have been allocated to the two distinct receptors, with vasoconstrictor activity designated to ET<sub>A</sub> (Fried et al., 1991). Endothelins have thus been implicated in a number of uterine functions, firstly in mediating vasoconstriction before and after menstruation, to regulate blood loss, but also in facilitating decidualisation and implantation (Cameron et al., 1992; Marsh et al., 1995; Marsh et al., 1996; Kohnen et al., 1997).

#### **1.14 Implantation**

Successful implantation is crucial for the establishment of pregnancy (Edwards, 1995). It involves a distinct series of events, between a perfectly synchronised embryo and endometrium, beginning with fertilisation of the oocyte to the formation of a fully functional placenta (Tabibzadeh et al., 1995a). The embryo prepares for implantation during cleavage. Sufficient divisions must have occurred to create adequate cell numbers to form the inner cell mass and trophoctoderm. Embryonic transcription is initiated at the 8 cell stage and after hatching from the zona pellucida on day 7 (Edwards et al., 1978), partial differentiation of the cytotrophoblast cells to syncytiotrophoblast takes place (Bevilacqua et al., 1988). Trophoblast cells are the source of numerous growth factors, inducers, and cytokines, which are believed to assist in the preparation of the uterus for implantation (Edwards, 1995) and adhesion molecule expression has been detected (Sutherland et al., 1993).

As previously stated, synchronisation of embryo and endometrium is vital for successful implantation and to this end a putative "implantation window" has been identified during which the endometrium possesses maximal receptivity (Psychoyos, 1994). This interval of 4 days spans days 20 - 24 of the menstrual cycle and is characterised by the expression of a number of crucial factors. Firm adhesion of the blastocyst is believed to be mediated through the cell adhesion molecules integrins

and their counter receptors on the trophectoderm (Klentzeris, 1997; Sueoka et al., 1997). Integrins are heterodimeric transmembrane glycoproteins, comprised of an  $\alpha$  and  $\beta$  subunit (Klentzeris, 1997). They interact with components of the ECM and cell surface molecules, facilitating cell-cell communication, cell attachment and migration. Cycle dependent expression of 3 integrin dimers  $\alpha_1\beta_1$  (collagen / laminin receptor),  $\alpha_4\beta_1$  (fibronectin receptor) and  $\alpha_v\beta_3$  (vitronectin receptor) in endometrial glandular and surface epithelium has been identified, the co-expression of which frame the implantation window (Lessey et al., 1994). These have proved to be reliable markers of endometrial receptivity, for instance, in circumstances of luteal phase defect (LPD), a disorder characterised by a delay in endometrial maturation and related to infertility, anomalous integrin expression has been reported (Lessey et al., 1996). Administration of steroid hormones in the form of the oral contraceptive alters integrins associated with uterine receptivity (Somkuti et al., 1996). In addition,  $\alpha_4\beta_1$  (also named very late antigen-4 (VLA-4)), which is proposed to interact with fibronectin expressed by trophoblast, is undetectable in women with unexplained infertility (Klentzeris et al., 1993).

Another marker of endometrial receptivity which has emerged in recent years is leukaemia inhibitory factor (LIF) (Stewart, 1994). This pleiotropic cytokine, affecting proliferation and differentiation of variety of cell types, is strongly expressed by the glandular epithelium in the mid secretory phase, temporally coinciding with the onset of the implantation window (Charnock-Jones et al., 1994). Furthermore, in transgenic mice homozygous for LIF deficiency (LIF<sup>-</sup>/LIF<sup>-</sup>), implantation failed to occur (Stewart et al., 1992). Preimplantation embryos were unaffected as indicated by successful implantation when transferred to wild type recipient mothers and by the "rescue" of implantation in LIF<sup>-</sup>/LIF<sup>-</sup> female mice by exogenous LIF injection. The role LIF plays in human implantation is not yet understood, but factors negatively influencing endometrial receptivity due to retardation of endometrial development (Gemzell-Danielsson et al., 1997), such as administration of 200mg or 400mg of the antigestogens mifepristone (RU486) and onapristone (ZK98299) immediately postovulation (LH+2), result in diminished LIF immunoreactivity in glandular epithelium on day LH+6 (Cameron et al., 1997). Similarly, intermittent daily low dose administration of mifepristone reduces LIF immunostaining in mid luteal phase glandular epithelium (Gemzell-Danielsson et al., 1997a).

The process of implantation has been likened to an inflammatory response, with common features including increased vasopermeability, leukocyte accumulation and

expression of inflammatory mediators (Kelly et al., 1994). All these factors are believed to have an integral role in the promotion and modulation of trophoblast invasion. Of particular interest are the locally acting mediators, interleukin-1 (IL-1) and prostaglandins (PGs). The IL-1 system has been implicated in the initiation of implantation, following studies in mice whereby blockade of IL-1 activity, by exposure to the naturally occurring receptor antagonist, prevents attachment and implantation of the embryo (Simon et al., 1994). The IL-1 system is comprised of the 2 ligands, IL-1 $\alpha$  and IL-1 $\beta$  (which share 26% homology), IL-1 receptors (types 1 and 2) and the previously mentioned receptor antagonist (IL-1ra). IL-1 type 1 receptor (IL-1R t1) is expressed by most cell types and appears to be the major route of signal transduction (Polan et al., 1995). IL-1 $\beta$  is produced and secreted by stromal macrophages and vascular endothelial cells of the luteal phase endometrium and additionally by the peri-implantation embryo (Simon et al., 1993). The expression of the receptor by glandular and surface epithelium during the implantation window furthers the supposition that IL-1 is involved in embryo-endometrium interactions (Simon et al., 1995a). IL-1ra has also been detected in the human endometrium as a product of epithelial cells and circulating monocytes (Simon et al., 1995b), as both intracellular (Haskill et al., 1991) and secreted (Eisenberg et al., 1990) forms. Thus complex interplay between all members of the IL-1 system is likely to be a key element in regulating implantation.

The involvement of prostaglandins in implantation remains uncertain, although their temporal expression and specific actions on decidualisation and the vasculature strongly reinforce an association (Psychoyos et al., 1995). Prostaglandins (PGs) are produced primarily by the glandular epithelial cells (Smith et al., 1988) and high concentrations of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  have been detected in the peri-implantation window and during menstruation (reviewed by (Van der Weiden et al., 1991)). Progesterone regulated PGE binding sites have been recognised in stromal cells (Kennedy et al., 1983a; Kennedy et al., 1983b), which are downregulated following exposure to RU486 (Martel et al., 1989). PGs have been primarily implicated in the extensive vascular changes occurring immediately prior to implantation (Kennedy et al., 1981). The dramatic increase in vascular permeability is essential for both implantation and decidualisation, and administration of PG inhibitor indomethacin inhibits or delays these events (Lau et al., 1973; Rankin et al., 1979). Moreover, PGE treatment of cultured stromal cells induces decidualisation (Kennedy, 1986). In the mouse, the PGE<sub>2</sub> receptor (EP2 subtype, through which the vasodilatory effects of PGE<sub>2</sub> are mediated) is selectively expressed by the luminal epithelium at exactly the

time when implantation would take place (Lim et al., 1997a). It is therefore evident that a complex network of locally acting factors exists, interacting to promote and regulate implantation.

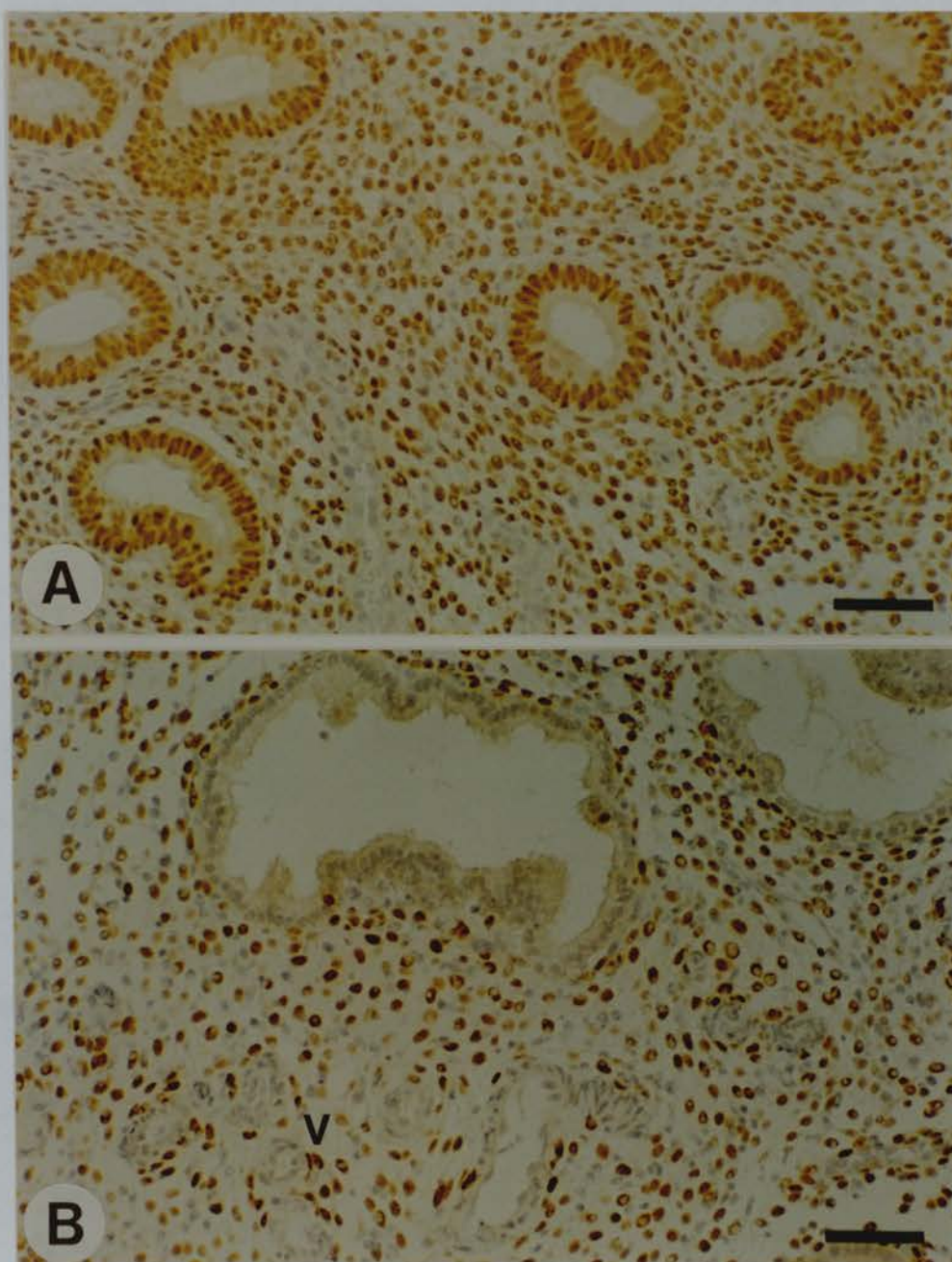
The first contact between embryo and endometrium takes place around 5 - 6 days after fertilisation, during which the blastocyst has travelled down the fallopian tube reaching the uterine cavity. The initial contact between embryo and endometrium is almost certainly mediated or enhanced by the presence of pinopodes on the surface epithelium of the endometrium (Nikas, 1995). These organelles have been implicated in the pinocytotic reduction of uterine fluid volume which facilitates close contact between the trophoblast and epithelium (Edwards, 1995). Pinopodes are clearly visible by ultrastructural observation of mid-luteal phase endometrium (days 19 - 21) (Martel et al., 1981) corresponding to peak progesterone levels (Martel et al., 1991). A suggestion that pinopodes may also express cell adhesion molecules has raised further interest in their role in blastocyst attachment (Kimber, 1994).

Apposition of embryo and epithelium produces the initial contact and leads to a further stronger attachment, thought to be mediated through integrins and other cell adhesion molecules (CAMs) (Klentzeris, 1997; Sueoka et al., 1997). Newly differentiated syncytiotrophoblast penetrates the surface epithelium, and causes the degradation of the underlying basement membrane. Further invasion of the endometrium functionalis takes place, directed towards the specialised spiral arterioles. The endometrium and basal lamina are systematically degraded as the trophoblast makes firm contact with the maternal arteries. Once sufficiently bathed in maternal blood, the cytotrophoblast cells migrate within the syncytiotrophoblast, and rapid angiogenesis ensues providing the placenta proper with an extensive vascular network. The highly invasive implantation occurring in humans requires careful modulation, as over- or under-invasion can lead to a number of clinical complications, such as placenta accreta, where excessive invasion of the myometrium and peritoneum occurs resulting in maternal haemorrhage; and disorders ranging from pre-eclampsia or intrauterine growth retardation to fetal death with insufficient placentation (Pijnenborg, 1994). There appears to be a finely tuned balance, probably mediated by the production of numerous local factors by both the peri-implantation endometrium and the lymphoid cells closely associated with invading trophoblast (Simon et al., 1996). These will be discussed in more detail in later sections of the introduction (section 1.3).



## 1.2 Sex Steroid Hormones

The ovarian sex steroid hormones, oestradiol and progesterone are responsible for the broad cyclical changes in endometrial histological appearance, as well as in the regulation of many paracrine factors. Signal transduction occurs through their interaction with intranuclear receptors, transcription factors which bind to specific palindromic hormone response elements (HRE) located in the promoter regions of target genes, positively or negatively regulating gene expression. Oestrogen (ER) and progesterone receptors (PgR) belong to a superfamily of nuclear steroid receptors including glucocorticoid, mineralocorticoid and androgen receptors, and share many functional similarities (Catt, 1996). In particular, in reproductive tissues, progesterone binding to PgR is likely to effect transcriptional activity in a similar fashion to cortisol (Strahle et al., 1989). The expression of the receptors is tightly regulated by the steroids themselves, with oestradiol inducing transcription and translation of both ER and PgR, and progesterone causing their downregulation (Clarke et al., 1990). A definitive pattern of steroid receptor expression in the endometrium has been reported (Lessey et al., 1988; Snijders et al., 1992) and proves to be a useful tool in assessing endometrial maturation (Garcia et al., 1988; Bouchard et al., 1991). ER are strongly expressed by glandular and stromal elements during the proliferative phase, with peak expression prior to ovulation in the late proliferative stage. Downregulation by progesterone occurs in the secretory phase in all cell types, resulting in negligible ER presence. Similarly, PgR expression is elevated in both glandular epithelial and stromal cells in the proliferative phase and in the immediate postovulatory period. Glandular receptors diminish with exposure to progesterone and are undetectable during the remainder of the menstrual cycle. PgR do persist, albeit at a lower level, in the stromal compartment. It is interesting that in the secretory phase, PgR are particularly apparent around the blood vessels (see Figure 2), especially the spiral arterioles (Bouchard et al., 1991; Perrot-Applanat et al., 1994). There is controversial evidence for the expression of PgR by the endothelial cells, with positive immunostaining detected only during pregnancy (Bouchard et al., 1991; Wang et al., 1992; Perrot-Applanat et al., 1994). This spatial expression pattern is maintained throughout pregnancy, with a possible shift from nuclear to cytoplasmic localisation of PgR proposed in third trimester decidua (Wu et al., 1991).



**Figure 2** Progesterone receptor distribution in the (A) proliferative and (B) secretory phase endometrium. Immunostaining using a monoclonal antibody specific for PgR<sub>A</sub> and PgR<sub>B</sub> isoforms was conducted by Dr Hong Wang. Note the presence of PgR in perivascular cells of blood vessels (V) in secretory phase endometrium.

Recently a second isoform of both the ER and PgR has been discovered. A novel ER, named ER- $\beta$ , was cloned in 1996 from rat prostate and ovary (Kuiper et al., 1996) and possesses a high sequence homology cDNA with the existing ER, now named ER- $\alpha$  (Gustafsson, 1997). Both isoforms of the ER exhibit similar affinities and specificities for oestrogens, and can induce transcription of an oestrogen responsive reporter gene construct in an identical fashion. A recent publication reveals that ER- $\alpha$  and ER- $\beta$  can form transcriptionally active heterodimers (Pettersson et al., 1997). It is believed that differences in the expression of the two isoforms determine the regulation of target genes in different tissues. There appears to be a degree of overlap in function, however, as the ER- $\alpha$  knock out mouse described in 1993 possessed no gross histological abnormalities, although multiple reproductive abnormalities were detected (Lubahn et al., 1993). The expression of ER- $\beta$  has been reported in various tissues, including the ovary and uterus (Byers et al., 1997; Deb et al., 1997), although no detailed studies into endometrial expression have been conducted to date.

The progesterone receptor exists in the A and B isoforms, following the discovery of the B form with an 164 amino acid N-terminal extension (Tung et al., 1993). PgR<sub>A</sub> (94kDa) and PgR<sub>B</sub> (120kDa) proteins arise from translation of separate mRNAs due to different transcription initiation sites on the same gene (Kastner et al., 1990). Both isoforms bind to progesterone and elicit a positive response (Shrader, 1993). Differences in signal transduction and cellular response have been suggested (Kastner et al., 1990), although this has not been confirmed and the implications for endometrial physiology are equally uncertain. It has been reported that PgR<sub>A</sub> and PgR<sub>B</sub> isoforms can form heterodimers and interact with progesterone response elements, with potentially different transcriptional activation properties than AA and BB homodimers (Meyer et al., 1990). An interesting finding is that in certain situations antigestogen binding to PgR<sub>B</sub> elicits a positive response, i.e. acts as a partial agonist (Meyer et al., 1990). Both isoforms of PgR are present in human endometrium, with peak expression of both in the mid proliferative phase (Wang et al., 1997b). The ratio of PgR<sub>A</sub>:PgR<sub>B</sub> was reported to be increased premenstrually and immediately after menses (Mangal et al., 1995). PgR<sub>B</sub> appear to be preferentially downregulated by progesterone in the secretory phase and in early pregnancy, whilst PgR<sub>A</sub> are the dominant form in secretory and decidual stromal cells (Wang et al., 1997b). The differential regulation of the two forms is further supported by the findings that in progestin exposed endometrium (via a levonorgestrel releasing intra-uterine device (LNg-IUS) or subdermal administration



(Norplant<sup>®</sup>)) PgR<sub>A</sub> are the predominant isoform (Critchley et al., 1998a; Wang et al., 1997a).

Modulation of progesterone levels is a useful tool in reproductive medicine. Since the discovery of antiprogestins, a greater understanding of progesterone function has been attained, manifesting itself in the use of the receptor antagonists for a variety of clinical and biological uses. A common use for mifepristone (RU486) is in the medical termination of pregnancy (< 9 weeks gestation), in combination with a dose of a prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) analogue (Cameron et al., 1986a; Bahzad et al., 1989). Investigations into the contraceptive potential of mifepristone or closely related onapristone are continuing, based on the theory that administration of antiprogestins delay or disrupt uterine development, thus interfering with endometrial receptivity (Cameron et al., 1994; Gemzell-Danielsson et al., 1994).

The precise molecular and cellular actions of the steroid hormones are not fully realised. For instance, it is not clear whether the effects on endometrial morphology are conveyed directly through binding to the nuclear receptors in target cells, or indirectly through steroid responsive cytokine networks. The latter situation is certainly supported by the obvious steroid mediated changes and events occurring in cell types when receptor expression is minimal (e.g. induction of epithelial gland activity in the secretory phase).

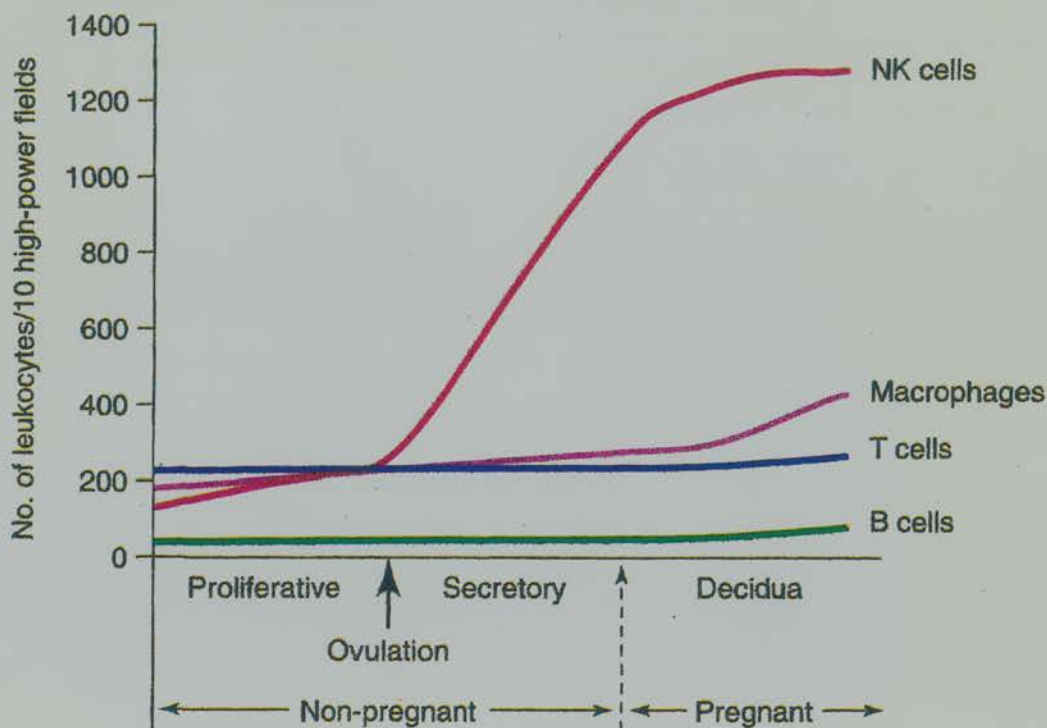
### **1.3 Endometrial Leukocytes**

The presence of lymphoid cells in the endometrial stroma has been well documented. Fluctuations in the number and subtype of endometrial leukocytes have been reported throughout the menstrual cycle and in pregnancy (Bulmer et al., 1991a; Starkey et al., 1991). In the proliferative phase, few leukocytes are detectable in the endometrium. A significant increase in the leukocyte numbers is observed in the mid to late secretory phase and these remain during early pregnancy (Figure 3). The majority of endometrial leukocytes are the phenotypically unique large granular lymphocytes (LGLs, eGLs), which are specific to the uterus and macrophages. In addition, a small number of T lymphocytes are present scattered throughout the stroma and surrounding epithelial glands, but no cyclical variations in number have been observed. Mast cells have been identified scattered throughout the stroma, with obvious peaks in activation in the mid-secretory and perimenstrual phase (Jeziorska et al., 1995). In early pregnancy, macrophages and LGLs are the predominant leukocyte subpopulations in the uterus (Loke et al., 1995a; Loke et al., 1997), but a



rapid decline in LGL numbers occurs by the third trimester of pregnancy, resulting in their virtual absence at term (Bulmer et al., 1991a; Haller et al., 1993). In the absence of pregnancy, a distinct influx of neutrophils and eosinophils is observed in the immediate premenstrual phase (Poropatich et al., 1987; Jeziorska et al., 1995).

Macrophages form around 20% of the total endometrial leukocyte population in late secretory endometrium and first trimester decidua (Loke et al., 1995a). Originally no fluctuations in cell number throughout the cycle were identified (Bulmer et al., 1988), but recently the availability of monoclonal antibodies with perhaps greater specificity for macrophages (e.g. CD68, CD14) has resulted in opposing findings. In particular, an increase in macrophage numbers has been noticed from the mid-late luteal phase and in first trimester decidua (Kamat et al., 1987; Klentzeris et al., 1992).



**Figure 3** Changes in leukocyte subpopulations throughout the menstrual cycle and in first trimester of pregnancy. The major subtype are the uterine specific LGLs (uterine NK cells), the numbers of which increase sharply in the secretory phase and in early pregnancy. Elevated numbers of macrophages are present in the secretory phase and a further increase is observed in early pregnancy. No significant fluctuations in the number of T or B cells is detectable. *Reproduced, with permission, from Loke, Y.W. and King, A., Mol Med Today 3, 153-159*

LGLs are believed to be of the natural killer (NK) cell lineage (Dietl et al., 1992) due to their strong expression of cell surface antigen CD56 (embryonic neural-cell adhesion molecule E-NCAM) (Lanier et al., 1989), which is expressed to a lesser extent by peripheral circulating NK cells (King et al., 1991). However, other classical NK markers, including CD16 and CD3 are absent. LGLs exhibit reduced cytotoxic activity towards the K562 cell line, a traditional NK cell target (King et al., 1989). The favoured hypothesis as to the mechanism of LGL appearance in the endometrium is that a precursor cell type is selectively recruited to the endometrium from the peripheral circulation, whereby it is activated to become the uterine specific LGLs (Marzusch et al., 1993). This is supported by the existence of a subset of circulating NK cells, which have low surface expression of CD56 and no CD16 expression. These comprise around 1% of peripheral NK cells (Lanier et al., 1986), and elevated numbers have been detected in women (King et al., 1991). Adhesion molecules capable of facilitating LGL / endothelial interactions have been identified on decidual blood vessels and LGLs, particularly ICAM and CD11a ( $\alpha_L\beta_2$ ) and to a lesser degree VCAM and counter receptor CDw49a ( $\alpha_4\beta_1$ ) (Marzusch et al., 1993), in agreement with previous examinations of NK cell migration (Allavena et al., 1992). It has been proposed that upon entry to the endometrium, a precursor cell type could undergo proliferation and differentiation, with the acquisition of granulated cytoplasm and increased CD56 cell surface expression (King et al., 1991). Treatment of the putative precursor cells with IL-2 upregulates cell surface CD56 expression and produces a decidual LGL-like phenotype (Dietl et al., 1992; Saito et al., 1993a). Other potential candidates which may be responsible for such an alteration have been proposed, including C-C chemokines (Maghazachi et al., 1996), transforming growth factor- $\beta_2$  (TGF- $\beta_2$ ) (Saito et al., 1993a), PGE (Linnemeyer et al., 1993), IL-12, IL-15, and prolactin (Handwerger et al., 1992; King et al., 1992; Loke et al., 1995b), although no conclusive evidence has been offered.

The exact function of the endometrial population of leukocytes is unknown. Both the LGLs and macrophages, however, are believed to be an important component of the decidualised endometrium, vital for the regulation of implantation and placentation (Bulmer et al., 1991b; King et al., 1991). Several studies have examined the distribution of leukocytes in decidua collected from women suffering recurrent miscarriage. Although a major cause of spontaneous abortion is fetal genetic abnormality, between 40-60% of cases remain unexplained (Stray-Pederson et al., 1984). A link between uterine leukocytes and unsuccessful pregnancy was forged following the detection of elevated Th1-like (inflammatory) cytokine expression by

decidual leukocytes derived from cases of recurrent abortion (Hill et al., 1995). This is believed to create a hostile environment to the developing fetus, and successful pregnancy has been related to a Th2 profile in the uterus (Lin et al., 1993)(see section 1.54). Alterations in endometrial leukocytes have been described in women suffering from failed pregnancy, including increased ratios of CD4<sup>+</sup>:CD8<sup>+</sup> T cells, elevated numbers of B cells and classical NK cells (CD57<sup>+</sup>) and also an overall reduction in total leukocyte numbers (Lachapelle et al., 1996; Vassiliadou et al., 1996).

LGLs cluster near glands and spiral arterioles, thus experiencing a close spatial relationship with decidualised stromal cells and invading trophoblast (King et al., 1990; Bulmer et al., 1991b). A role in the modulation of trophoblast invasion and protection of the genetically distinct fetus has been postulated. Rejection of foreign transplanted grafts involves T cell recognition of highly polymorphic major histocompatibility complex (MHC) antigens (human lymphocyte antigens (HLA)). HLA Class 1 antigens are expressed by nearly all nucleated cells, and may be subdivided into classical (HLA-A, -B and -C) and non classical (HLA-E, -F and -G) families. Extravillous trophoblast expresses both HLA-C and -G (Ellis et al., 1990; Kovats et al., 1990; King et al., 1996a), both of which exhibit low polymorphism and surface expression. The prime recognition target cell is believed not to be T cells, but the uterine specific LGLs (Loke et al., 1997), reinforcing the evidence for a primitive immunological system in the uterus (Loke et al., 1996). Peripheral NK cells interact with HLA-C and -G via three distinct receptor families. The KIRs (killer inhibitory receptors) and KARs (killer activator receptors) stimulate opposing responses, through cytotoxicity and cytokine production (Gumperz et al., 1995; Lanier et al., 1996). The third receptor designated CD94 (Philips et al., 1996) is believed to bind to both HLA-C and -G, and to have a similar role in the modulation of cell killing and cytokine production. Initial investigations into the expression of KIRs / KARs and CD94 by decidual LGLs support the hypothesis that LGLs interact with the non-classical and classical HLA antigens (Loke et al., 1997). NK cells preferentially kill cells with little or no surface expression of class I HLA antigens (the "missing self hypothesis") (Ljunggren et al., 1990), thus their presence would be protective to the trophoblast (Pasmany et al., 1996). A balance is likely to exist between LGL mediated cell killing and protection depending on the trophoblast expression of HLA-C and -G and the repertoire of maternal NK receptors (Loke et al., 1997).

A further element of regulation of implantation and menstruation is through the

ability of the LGLs, macrophages, and to some extent neutrophils, to produce a vast array of immunoregulatory factors including cytokines and prostaglandins (Haller et al., 1993; Saito et al., 1993b; Leslie et al., 1994; Saito et al., 1994). Additionally, stromal macrophages, neutrophils, eosinophils and mast cells may also have a function in tissue breakdown or remodelling in the perimenstrual and menstrual period. All have the potential to produce MMPs and additionally, contain potent activators of endometrial derived MMPs (mast cell tryptase, neutrophil elastase, granzyme A, perforin etc.) (King et al., 1993; Salamonsen et al., 1996b). It has also been proposed that these enzymes could induce trophoblast cell death (Shiver et al., 1992), possibly in cases of abnormality or over invasion, and additionally can affect migration and proliferation by altering attachment to the ECM. A role can therefore be visualised for endometrial leukocytes both in the promotion and regulation of implantation and as contributors to the tissue breakdown and the inflammatory response which occurs prior to and during menses.

The factors controlling the mode of appearance and fluctuation of leukocyte subtypes throughout the menstrual cycle are similarly uncertain. The cyclical pattern of leukocyte presence is suggestive of steroidal control, but the recent demonstration that they do not possess either ER or PgR implies that this regulation is exerted indirectly (King et al., 1996b). In a sheep model, an influx of leukocytes occurs on withdrawal of progesterone (Staples et al., 1983). It is interesting that the accumulation of endometrial leukocytes in the human corresponds to decreasing steroid levels, leading to the hypothesis that progesterone presence allows decidualisation and suppresses leukocyte infiltration (Clemens, 1977). Withdrawal of progesterone stimulates an acute inflammatory response, including leukocyte accumulation, and culminates in menstruation (Finn, 1986). Co-localisation studies have revealed that in the secretory phase of the menstrual cycle stromal leukocytes express the cell proliferation marker Ki67 (Pace et al., 1989; Tabibzadeh, 1990; King et al., 1991) indicating that the increase in numbers is accounted for, at least in part, by *in situ* proliferation. However, the further increase in LGL numbers observed in first trimester decidua can not be accounted for by proliferation. It is likely however, that the migration of leukocytes from the peripheral circulation also contributes to leukocyte accumulation (Lea et al., 1991; Marzusch et al., 1993).

#### **1.4 Leukocyte Migration**

Leukocyte migration from the peripheral circulation into tissue stroma occurs at sites of inflammation. This is a complex multistep event, involving sequential leukocyte



adherence, simultaneous expression of leukocyte adhesion molecules and their corresponding receptors on endothelial cells (Frenette et al., 1996), and directed migration along a chemotactic gradient (Adams et al., 1994; Schall et al., 1994; Springer, 1990; MacKay and Imhof, 1993). For description purposes, this can be separated into 3 main steps:

1. An initial loose contact between leukocyte and the endothelial cells lining the vessel is mediated by the cell adhesion molecules, selectins. E-selectin / P-Selectin expression on the luminal endothelium is up-regulated by pro-inflammatory mediators, such as IL-1 $\beta$  and TNF- $\alpha$ , stimulating endothelial activation. Leukocytes may then bind via L-Selectin, which contains the carbohydrate sialyl Lewis X ligand. This forms a transient and reversible bond, permitting "rolling" of the leukocyte along the endothelial cell surface. This prolonged contact with the endothelial cells is believed to enhance exposure of the leukocyte to chemoattractants, and the leukocyte itself may produce mediators to further activate the endothelium.

2. Rolling is arrested by a secondary activation of integrins on the surface of leukocytes by an alteration of the extracellular binding domain. These are of the  $\beta$ 2 and  $\beta$ 1 integrin family which include lymphocyte function-associated antigen - 1 (LFA-1) and very late-activation antigen-4 (VLA-4). These allow the formation of a firm attachment of the leukocyte via counter receptors such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) respectively. In addition, platelet endothelial cell adhesion molecule (PECAM) has been identified in the endometrium. These cell adhesion molecules are also up regulated by the release of proinflammatory mediators IL-1 $\beta$ , TNF- $\alpha$ , Interferon- $\gamma$  (IFN- $\gamma$ ) and chemokines. Leukocytes become tightly adhered to the vessel wall and take on a flattened morphology.

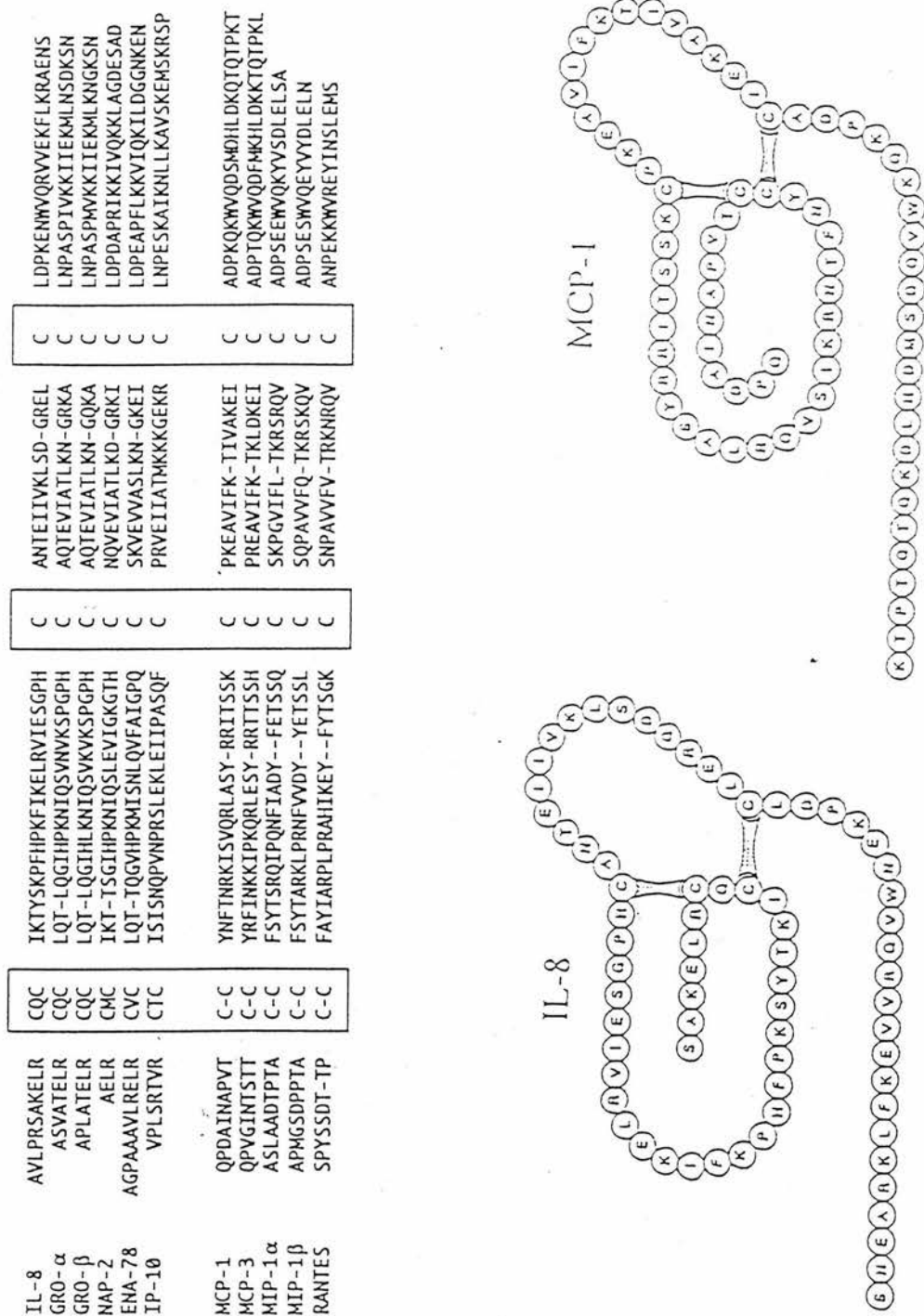
3. Transendothelial migration may now occur, by the extravasation of leukocytes through cell junctions into the extracellular matrix of underlying tissue. Migration of the leukocyte into a particular tissue and to a specific location within the stroma is directed by locally produced chemotactic agents. The transendothelial gradient is generated by the immobilisation of chemoattractants by components of the ECM. Glycosaminoglycans (GAGs) on the proteoglycan CD44, particularly chondroitin sulphate, have been demonstrated to form a scaffolding for C-C chemokine macrophage inflammatory protein- $\beta$  (MIP-1 $\beta$ ) (Tanaka et al., 1993; Koopman et al., 1997) and a similar mechanism has been proposed for sequestering interleukin-8 (IL-

8) and related chemokines (Witt et al., 1994; Middleton et al., 1997). Chemokines have the potential to facilitate or amplify leukocyte infiltration, by the upregulation of adhesion molecules (Jonjic et al., 1992).

## **1.5 Inflammatory Mediators**

### **1.51 Chemokines**

A family of cytokines which possess specific chemoattractant activity for leukocyte subtypes are the chemokines (for reviews (Matsushima et al., 1992; Baggiolini et al., 1994; Mantovani et al., 1996; Baggiolini et al., 1997)). Chemotactic cytokines or chemokines play a major role in the recruitment of inflammatory cells to sites of infection, thus being potent mediators of inflammatory responses. Much interest has been raised into the expression of chemokines, due to their involvement in many pathological states, including asthma, rheumatoid arthritis, allergies and atherosclerosis (Gura, 1996; Strieter et al., 1996). The chemokine family comprises a number of structurally related low molecular weight heparin binding glycoproteins (8 - 10 KDa) sharing 20 - 80% homology at the amino acid (aa) level. Members of the chemokine family are identified by the presence of four highly conserved cysteine residues. The positioning of the first two N-terminal cysteine residues determines the classification into one of two subfamilies (Lindley et al., 1993). The  $\alpha$  or C-X-C chemokines are characterised by an intervening unconserved amino acid residue between the cysteine pair. Conversely, the members of the  $\beta$  or C-C family are recognised by the adjacent positioning of the cysteine residues. The recent discovery of a novel chemokine, lymphotactin, which does not fit into either previously designated family, has caused the alteration in the nomenclature to include a third subfamily (Kelner et al., 1994). This comprises to date just the single chemokine, with the distinctive structural motif of a single cysteine residue at the N-terminal region (hence the nomenclature as the C or  $\gamma$  chemokine subfamily). Lymphotactin is chemotactic for T and B lymphocytes but not monocytes or neutrophils. Earlier this year a further family of chemokines, the fractalkines, possessing the C-X-X-X-C motif, were described (Bazan et al., 1997; Schall, 1997). The number of members and the complexity of the chemokine superfamily continues to expand with increasing research into this field (Yoshie et al., 1997).



**Figure 4** Amino acid sequences of representative CXC and CC chemokines. Sequences are aligned to the four highly conserved cysteine residues, shown in boxes. Secondary structures are also illustrated for IL-8 and MCP-1, showing disulphide bonds. Single letter amino acid codes: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (Modified from Baggiolini et al, 1994).



Interleukin-8 (IL-8) or neutrophil attractant protein-1 (NAP-1) is a classical chemokine, whose expression and activity has been extensively investigated. IL-8 belongs to the  $\alpha$  or C-X-C family and further members include melanocyte growth stimulatory activity / growth related oncogene- $\alpha$  (MGSA / GRO- $\alpha$ ), GRO- $\beta$ , GRO- $\gamma$ , neutrophil-activating protein derived from epithelial cells (ENA-78) and interferon-inducible protein (IP-10). Generally, all possess chemotactic activity for neutrophils, although certain members (IL-8 and IP-10) have been attributed with the induction of T lymphocyte chemotaxis (Larsen et al., 1989a; Taub et al., 1993). Further properties include activation of neutrophils, by inducing shape change and exocytosis. Thus neutrophils may be recruited to a site of inflammation upon which they are activated to release their repertoire of immunomodulatory cytokines and proteases. This infiltration is enhanced by PGE which acts synergistically in the migration of neutrophils into rabbit skin sites (Foster et al., 1989; Rampart et al., 1989; Colditz, 1990).

IL-8 was originally detected as a monocyte secreted product, but is now known to also be produced by a variety of cell types, including epithelial, endothelial and smooth muscle cells, fibroblasts and lymphocytes. The gene encoding IL-8 is situated on chromosome 4, amongst a cluster of C-X-C chemokine genes in the q12-21 region, and is comprised of 4 exons and 3 intronic regions (Matsushima et al., 1988; Mukaida et al., 1989). Upon induction by numerous pro-inflammatory mediators, e.g. IL-1, TNF- $\alpha$ , LPS, phorbol esters and certain viruses, the 297bp coding region is transcribed and translated producing a 99aa precursor protein. After cleavage of the 20aa leader sequence, the mature form is secreted and subjected to post translational processing of the N-terminal region. IL-8 exists as a homodimer in solution, although the monomeric form is the recognised active form (Rajaratnam et al., 1994).

Monocyte chemoattractant protein-1 (MCP-1) is a typical C-C chemokine. Unlike the C-X-C family, the  $\beta$  or C-C chemokines do not stimulate neutrophil chemotaxis, but instead possess chemotactic activity for mononuclear cells particularly monocytes. MCP-1 additionally is a potent chemoattractant for T lymphocytes (Cai et al., 1995; Roth et al., 1995), basophils, mast cells (Feliciani et al., 1995) and also natural killer cells (Allavena et al., 1994) and IL-2 activated NK cells (Maghazachi et al., 1994). Other members of the C-C chemokine subfamily are monocyte chemoattractant proteins-2, -3, -4 and -5 (MCP-2, MCP-3, MCP-4, MCP-5) (Godiska et al., 1997; Sarafi et al., 1997), eotaxin, macrophage inflammatory

protein-1 $\alpha$  and 1 $\beta$  (MIP-1 $\alpha$  and 1 $\beta$ ) and RANTES (Regulated on Activation, Normal T Expressed and Secreted) (Uguccioni et al., 1995; Uguccioni et al., 1996; Baggiolini et al., 1997). The genes encoding MCP-1 and other C-C chemokines are located on human chromosome 17, closely positioned within the q11-21 region (Shyy et al., 1990).

MCP-1, the human homologue of the murine JE (Yoshimura et al., 1989), is a 76aa basic heparin binding protein, displaying varying degrees of glycosylation (Jiang et al., 1990). MCP-1 is positively regulated by pro-inflammatory stimuli, such as IL-1 and TNF- $\alpha$ , as is IL-8 (Baggiolini et al., 1994), but is additionally induced by platelet derived growth factor (PDGF) (Zullo et al., 1985). This growth factor has no effect on IL-8 production, indicating that regulatory differences in the gene expression of these chemokines exists (Yoshimura et al., 1990). Originally detected as an immediate early gene produced by fibroblasts (Rollins et al., 1988), MCP-1 production by epithelial, endothelial, smooth muscle cells and a variety of lymphoid cells has since been reported (Larsen et al., 1989b; Yoshimura et al., 1989; Sica et al., 1990; Wang et al., 1991).

**Table 1** Table of main chemokines:

SUBFAMILY	CHEMOKINES	TARGET CELLS	FUNCTIONS
C-X-C ( $\alpha$ )	Interleukin-8 (IL-8)	Neutrophils T cells Endothelial cells	Chemotaxis, Superoxide & Granule release Angiogenesis
	Growth related oncogenes (GRO- $\alpha$ , $\beta$ and $\gamma$ )	Neutrophils Endothelial cells	Chemotaxis Angiogenesis
	Interferon-inducible protein-10 (IP-10)	T cells Natural killer cells Endothelial cells	Chemotaxis Cytolytic activity Angiogenesis
	Epithelial derived neutrophil attractant (ENA-78)	Neutrophils	Chemotaxis
C-C ( $\beta$ )	Monocyte chemoattractant protein-1 (MCP-1)	Monocytes T cells Mast cells Basophils Natural Killer cells	Chemotaxis, Superoxide release, Phagocytosis
	MCP-2, -3, -4, -5	Monocytes T cells Mast cells Eosinophils	Chemotaxis, Histamine release
	Regulated on activation, normal T- cell expressed and secreted (RANTES)	Monocytes T cells Eosinophils Natural killer cells Eosinophils Basophils Dendritic cells	Chemotaxis, histamine release
	Eotaxin	Eosinophils	Chemotaxis

SUBFAMILY	CHEMOKINES	TARGET CELLS	FUNCTIONS
C-C ( $\beta$ )	Macrophage inflammatory protein-1 $\alpha$	Monocytes T cells B cells Natural killer cells Mast cells Eosinophils Basophils Mast cells Dendritic cells	Chemotaxis Activation, Histamine release
	MIP-1 $\beta$	Monocytes T cells	Chemotaxis
C ( $\gamma$ )	Lymphotactin	T lymphocytes	Chemotaxis
C-X-X-X-C (Fractalkines)		Monocytes T lymphocytes	Chemotaxis

### 1.52 Chemokine Receptors

Chemokines elicit their response via interaction with G protein coupled receptors, possessing the classical 7 transmembrane domains (Murphy, 1994; Premack et al., 1996). G (guanine nucleotide) binding proteins are comprised of three subunits, the  $\alpha$ ,  $\beta$  and  $\gamma$ . Ligand binding stimulates guanosine triphosphate (GTP) binding and hydrolysis. Two high affinity IL-8 receptors exist, the IL-8RA which binds solely to IL-8, and IL-8RB with specific binding for IL-8 and other members of the C-X-C family (Horuk, 1994; Chuntharapai et al., 1995). A promiscuous IL-8 receptor present on erythrocytes (the Duffy antigen receptor for chemokines (DARC) or erythrocyte chemokine receptor - ECKR) (Hadley et al., 1997), also transduces a signal through interaction with C-C chemokines including MCP-1, and is believed to act as a clearance mechanism for circulatory chemokines (Neote et al., 1993). This antigen, initially identified as participating in *Plasmodium vivax* (malaria parasite) cell invasion (Horuk, 1994), has since been detected on a number of non-erythroid cell types, including endothelial cells of postcapillary venules (Hadley et al., 1994). This corresponds to the site of leukocyte trafficking and it has been suggested that chemokines are immobilised on the endothelium through binding to DARC, in a

similar fashion as chemokine binding to proteoglycans, thus providing another possible mechanism for chemokine presentation to circulating leukocytes (Hadley et al., 1997; Middleton et al., 1997).

Multiple C-C chemokine receptors have been identified, each with differing ligand specificity. CCR1 (formerly named CC CKR1 or MIP-1 $\alpha$  / RANTES receptor) interacts with MIP-1 $\alpha$ , RANTES, MCP-2 and -3 (Gao et al., 1993; Ben-Baruch et al., 1995). CCR2 is a high affinity receptor for MCP-1 (Neote et al., 1993), but may also bind to MCP-2, -3, and -4 (Combadiere et al., 1995; Uguccioni et al., 1996). Three additional receptors have been cloned CCR3 - 5, each recognising at least three C-C chemokines (Baggiolini et al., 1997).

Chemokine binding to virally encoded receptors has also been reported, such as the human herpes virus and cytomegalovirus encoded receptors. The physiological significance of this is unknown. Additional interest in chemokine receptors has been prompted by the finding that the C-C chemokine receptors act as a cell entry co-factor for HIV-1 (Feng et al., 1996) and MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES are suppressors of viral replication, functioning through blocking entry to the receptor (Cocchi et al., 1995). Furthermore, humans homozygous for an inactivating mutation of the *CCR5* gene appear to be resistant to HIV-1 (Gao et al., 1997). This has intensified the search for chemokine receptor antagonists which could have significant functions in inflammatory situations, other than viral infection.

### **1.53 Reproductive Significance of Chemokines**

The involvement of chemokines in pathological conditions has been well investigated, however there is increasing evidence for their roles in physiological situations. Chemokine expression in reproductive tissues has been examined with regard to a role in normal physiological functioning. Interleukin-8 (IL-8) has been detected in the human cervix (Barclay et al., 1993), endometrium (Arici et al., 1993; Critchley et al., 1994), chorio-decidua (Dudley et al., 1993) and the placenta (Saito et al., 1994). Elevated levels of IL-8 are detectable in amniotic fluid in cases of infection-associated preterm labour and chorioamnionitis, leading to the speculation that the chemokine is involved in the pathogenesis of these conditions. A role for IL-8 in normal parturition has also been proposed (Kelly et al., 1992; Kelly, 1996). Cervical ripening precedes parturition and is associated with a neutrophil infiltrate. It has been postulated that these are the major source of collagenases and other MMPs which are responsible for the softening and increased elasticity of the cervix.

Increased production of IL-8 in the cervix coincides with neutrophil influx prior to ripening at term (Osmers et al., 1995) and cervical ripening can be reproduced in rabbits and guinea pigs following treatment with IL-8 (Chwalisz et al., 1994; El Maradny et al., 1996). Furthermore, IL-8 has been implicated in the premenstrual infiltrate of neutrophils to the endometrium, where it has been postulated they have a vital role in the initiation of menstruation (Kelly et al., 1994). Supporting evidence for the latter hypothesis came from immunohistochemical studies, where IL-8 was localised to the perivascular cells of blood vessels in non-pregnant and pregnant endometrium (Critchley et al., 1994). In this position a role in facilitating recruitment of neutrophils can be conceived.

Induction of IL-8 mRNA and protein by IL-1 $\alpha$  and TNF- $\alpha$  in cultured endometrial and decidual stromal and epithelial cells has been observed (Arici et al., 1993). These findings lend further support to a cytokine network in the uterus. A suppression of IL-8 production by chorio-decidual cells (Kelly et al., 1994) and cervix (Ito et al., 1994) by progesterone *in vitro* has been demonstrated, acting through progesterone receptors present within the cell, binding to progesterone / glucocorticoid response elements in the promoter region of the gene. If a similar downregulation by progesterone occurs *in vivo*, the withdrawal of progesterone premenstrually would remove the inhibitory effect, resulting in an increased production of IL-8 and accompanying neutrophil influx. Little is known about the downregulation of MCP-1, with the exception of an inhibition by glucocorticoids in certain cell lines (Brach et al., 1992; Shyy et al., 1995). This could indicate a negative regulation by progesterone in reproductive tissues.

MCP-1 has not been directly linked with normal uterine function, but a role in the pathogenesis of endometriosis has been postulated. Peritoneal fluid collected from laparoscopically diagnosed endometriosis contains elevated levels of MCP-1 and MCP-1 production by endometriotic cells has recently been described (Akoum et al., 1996). These findings correlate well with the increased numbers of activated macrophages present in the lesions (Halme et al., 1983; Halme et al., 1987). Furthermore, another C-C chemokine, RANTES, which shares many functional similarities with MCP-1, has been implicated in the aetiology of this disease (Hornung et al., 1997). A role for MCP-1 in leukocyte recruitment to the normal endometrium has not been investigated. MCP-1 mRNA and immunoreactive protein has been detected in ovarian tumours, associated with infiltrating macrophages, which are thought to contribute to the aetiology of the disorder (Negus et al., 1995). A recent publication describes MCP-1 in follicular fluid and as a product of ovarian



stromal cells (Arici et al., 1997). Ovulation is an inflammatory-like response, and a considerable population of macrophages is present in the follicle prior to rupture. A role in the process of ovulation through macrophage recruitment was substantiated by an elevation in MCP-1 levels both after hCG / LH administration and in *in vitro* culture systems.

Chemokines also possess properties not associated with leukocyte chemotaxis. IL-8 and MCP-1 are potent activators of neutrophils and monocytes, NK cells and lymphocytes respectively. Activated leukocytes undergo shape changes, produce an array of active cytokines, including chemokines, and prostaglandins, and may release degradative enzymes (Loetscher et al., 1996). A system whereby the local inflammatory response is amplified can thus be visualised, and further leukocyte homing is enhanced by upregulation of cell adhesion molecules. C-C chemokines have also been described to induce T cell proliferation and cytokine production (Alam, 1997). Of potential relevance in the endometrium, C-C chemokines, including MCP-1, can induce killer cells from CD56<sup>+</sup> NK cells, in a manner similar to IL-2 generation of LAK cells (Maghazachi et al., 1996). An independent research group have provided complementary results, of NK cell activation, enzyme release and Ca<sup>2+</sup> mobilisation by C-C chemokines (Loetscher et al., 1996).

A further example is that IL-8 and other C-X-C chemokines containing the E-L-R (glutamic acid-leucine-arginine) motif in their amino acid sequence, are potent stimulators of angiogenesis (Strieter et al., 1995a). Conversely, members of the C-X-C chemokine family that do not contain the E-L-R motif are potent inhibitors of angiogenesis, e.g. platelet factor 4 (PF4). IL-8 stimulates both endothelial and smooth muscle cell chemotaxis and proliferation (Koch et al., 1992; Yue et al., 1994). A role for chemokine induced angiogenesis has been determined in tumour associated vascularisation (Strieter et al., 1992; Strieter et al., 1995; Arenberg et al., 1997), and in neovascularisation of ectopic endometrium associated with endometriosis (Ryan et al., 1995) and a similar involvement in endometrial blood vessel development could be envisaged. The endometrium is one of the few adult non-malignant tissues in which angiogenesis regularly takes place (Goodger et al., 1994). Three peaks in endometrial angiogenesis have been identified (Rogers et al., 1992), for the regeneration of the functionalis vasculature following menses, in the mid proliferative phase under oestrogenic control and in the secretory phase whilst spiral arteriole differentiation occurs in the decidualised endometrium (Kaiserman-Abramof et al., 1989). There has been considerable evidence for the involvement of locally active factors in endometrial vessel growth, especially vascular endothelial

growth factor (VEGF) (Smith, 1996). Evidence of elevated peritoneal concentrations of IL-8 corresponding to neovascularisation in endometriotic lesions (Ryan et al., 1995) supports the hypothesis that this chemokine participates in angiogenesis in the endometrium.

### 1.54 Cytokines

A role for chemokines in the endometrium can thus be proposed, based on previous evidence of chemokine and endometrial functions. It must be remembered, however, that these are just a few components of a widespread and complex network of locally acting factors which together regulate cellular communications. First identified as products and modulators of immune cells, it is now clear that cytokines play a major role in the regulation of many physiological and pathological processes in virtually every cell type. Cytokines are capable of stimulating responses via intercrine, autocrine, paracrine and endocrine communications. Moreover, they have been widely recognised as vital mediators in important uterine processes, such as proliferation, decidualisation, implantation and menstruation, and further provide a means of communication between embryo and endometrium (Tabibzadeh, 1991; Tabibzadeh et al., 1995a). Cytokines rarely function in isolation, and thus a complex interplay of local factors is likely to contribute to cell signalling in most biological situations. This creates a great deal of overlap in functions, and introduces redundancy into the system. The cytokine family encompasses many divisions, the interleukins (1 - 15), interferons ( $\alpha$ ,  $\beta$  and  $\gamma$ ), colony stimulating factors (M-CSF, C-CSF, GM-CSF), tumour necrosis factors (TNF)  $\alpha$  and  $\beta$ , growth factors (epidermal growth factor (EGF), transforming growth factors (TGF), platelet derived growth factor (PDGF)). The list is too extensive to examine in full detail in this thesis, but cytokines with known importance in the hypothalamic-pituitary-ovarian-endometrial axis, with particular reference to the modulation of inflammatory type situations, will be discussed. Of particular interest are three pro-inflammatory mediators, IL-1, TNF- $\alpha$  and IL-6, all of which have been implicated in both normal and pathologically abnormal uterine functioning (Hill, 1992; Tabibzadeh, 1994a; Tabibzadeh, 1994b). Diverse functions including modulation of proliferation, leukocyte chemotaxis and activation, prostaglandin induction and initiation of menstruation have been attributed to these endometrial cytokines. The tight regulation of these factors is believed to be a key factor in successful reproduction (Austgulen et al., 1994).

Interleukin-1 (IL-1), as previously described (section 1.14), is a pro-inflammatory

mediator which has been implicated in numerous aspects of uterine function. Peaks of expression of IL-1 $\alpha$  and  $\beta$  are observed in the peri-implantation period and prior to menses. IL-1 functions primarily by inducing the transcription or increased translation of inflammatory factors, including IL-6, TNF- $\alpha$ , IL-8, MCP-1 and by induction of PGE synthesis (Romero et al., 1989). The detection of TNF- $\alpha$  and its two distinct receptors in the utero-placental unit has been well documented. This cytokine is produced by the secretory (Philippeaux et al., 1993) and menstrual phase endometrium (Tabibzadeh et al., 1994c) and villous trophoblast (Lea et al., 1997) and receptors are present in uterine cells. Previously conferred with chemotactic activity (Ming et al., 1987), this is likely to be an indirect effect via chemokine production (Matsushima et al., 1988). The expression of TNF- $\alpha$  by the endothelium of the endometrium (Philippeaux et al., 1993) reinforces roles in vascular permeability and induction of apoptosis (Robaye et al., 1991). Inappropriate expression of TNF- $\alpha$  appears to be detrimental to pregnancy, as demonstrated in a mouse model, where injection of the proinflammatory cytokine induced abortion (Chaouat et al., 1990). Additionally, elevated concentrations of both IL-1 and TNF- $\alpha$  have been identified in the peritoneal fluid of women with endometriosis (Fakih et al., 1987; Halme, 1989), in agreement with the inflammatory nature of this pathological disease, and further accounts for the increased levels of peritoneal chemokines.

IL-1 and TNF- $\alpha$  induce amongst other factors the pleiotropic cytokine interleukin-6 (IL-6). IL-6 in turn downregulates the expression of IL-1 and TNF- $\alpha$  (Chrousos, 1995), thus having an important role in the regulation of the inflammatory response. IL-6 is ubiquitously produced by a number of lymphoid and non-lymphoid cells, including the endometrial stroma, glandular epithelium and endothelium (Tabibzadeh et al., 1989a; Tabibzadeh et al., 1992; Laird et al., 1993), with a progressive rise in glandular and surface epithelium expression detected in the secretory phase (Tabibzadeh et al., 1995b). In a cell culture system, IL-6 production from stromal cells was shown to be inhibited by oestrogen at physiological concentrations (Tabibzadeh et al., 1989b), which correlates with its inhibitory effect on stromal cell proliferation (Zarmakoupis et al., 1995). IL-6 is a potent mediator of immune cell development, particularly T and B cell differentiation, and induces the synthesis of acute phase proteins (Gauldie et al., 1987). An involvement in folliculogenesis and regulation of ovarian steroidogenesis has been reported, and it has been postulated that IL-6 has a main role in the maintenance of endometrial homeostasis. Soluble and membrane bound forms of the receptor exist, which

dimerise with signal transducing glycoprotein gp-130. Increased serum concentrations of soluble receptor are observed during pregnancy (Austgulen et al., 1994). This has been correlated with a role in early placentation, placental hormone production (Nishino et al., 1990) and fetal immune system development (Biesecker et al., 1993). Recent evidence using knockout mice suggests that IL-6 also stimulates the expression of chemokines IL-8 and MCP-1, thus augmenting leukocyte infiltration into tissue sites (Romano et al., 1997).

Pioneering work by Wegmann provided the hypothesis of the Th1 / Th2 paradigm in the maternal-fetal interface (Wegmann et al., 1993). T helper cells (CD4<sup>+</sup>) may be classified into two groups, according to cytokine production and thus the type of immune response stimulated (Kurt-Jones et al., 1987; Mosman et al., 1989). It is now believed that T cells are not the sole sources of these cytokines, and a more general concept of Th1 and Th2 type cytokine production by most cell types is widely accepted (Mosmann et al., 1996). Th1 type cytokines (IL-2, IL-12 and IFN- $\gamma$ ) are inflammatory cytokines and generally stimulate a cell mediated cytotoxic or inflammatory response. Th2 type cytokines comprise IL-10, IL-4, IL-5, IL-6 and cause a switch to humoral (antibody) mediated responses. A Th2 profile has been postulated to confer local suppression of an inflammatory response and it has been proposed that a Th2 cytokine environment is favourable for successful allopregnancy (Wegmann et al., 1993). Indeed upregulation of IL-10 and other Th2 cytokines, is observed during pregnancy (Lin et al., 1993) and evidence for an upregulation of Th1 cytokines has been detected in failed pregnancy (Hill, 1995; Chaouat et al., 1990; Haynes and Smith, 1997). Th1 cytokines are believed to be detrimental to pregnancy, as TNF- $\alpha$ , IFN- $\gamma$  and IL-2 have all been demonstrated to have adverse effects on fetal development and trophoblast proliferation (Raghupathy, 1997). Furthermore, there is evidence for a alleviation of symptoms associated with Th1 type conditions during pregnancy (Da Silver et al., 1992; Krishnan et al., 1996).

Whilst T helper cells make up a minority of endometrial leukocytes, production of Th2-like cytokines by other lymphoid cells, in particular macrophages, and non-lymphoid endometrial cells and trophoblast could create this environment (Kelly and Critchley, 1997a). Prostaglandins are known to be important regulators of lymphocyte responses, and recently this has been reinforced by the suggestion that PGE<sub>2</sub> regulates the balance between humoral and inflammatory responses, through the favouring of a Th2 tilt (upregulation of IL-10 and downregulation of IL-12, IFN- $\gamma$ , IL-2) (Snijdwint et al., 1993; Strassmann et al., 1994; Hilkens et al., 1995; Kraan



et al., 1995). Furthermore, IL-10 selectively downregulates Th1 cytokines, especially IFN- $\gamma$  and IL-12 (Fiorentino et al., 1989; Mosmann et al., 1991). Both IFN- $\gamma$  and IL-12 are potent mediators of NK cell function, and thus may activate NK cells that may damage the trophoblast (Drake et al., 1989; Kelly et al., 1997a). The downregulation of Th1-type cytokines at the maternal-fetal interface during pregnancy may therefore decrease NK cell cytotoxicity and serve as a protective mechanism for trophoblast (Kelly, 1996). The hypothesis of the Th1 to Th2 switch during pregnancy remains controversial, however it is widely believed that there is a bias away from potentially harmful Th1 type cytokines and towards Th2 cytokine production in the pregnant uterus (Guilbert, 1996; Vince et al., 1996; Lim et al., 1998).

### **1.55 Nitric Oxide**

With increasing interest into paracrine interactions within the endometrium, attention has been focused on local mediators which may contribute to the processes of implantation, decidualisation and menstruation. Nitric oxide (NO) is a potent mediator of vascular permeability, immune cell function, and additionally has inhibitory actions on platelet aggregation and smooth muscle proliferation (Rosselli, 1997). NO is synthesised from L-arginine by nitric oxide synthase (NOS) in many distinct cell types. Three isoforms of NOS have been identified, two of which are constitutively expressed and are calcium dependent, endothelial NOS (eNOS) and neuronal NOS (nNOS), whilst the third, inducible NOS (iNOS), is rapidly induced in response to inflammatory type stimuli, independent of calcium.

In apparently divergent roles, NO has been identified to act as both an inflammatory and anti-inflammatory agent. Within the uterus, NOS has been detected in the glandular, stromal and vascular components of non-pregnant and pregnant endometrium, as well as in the myometrium (Telfer et al., 1995; Telfer et al., 1997). During pregnancy, additional NOS mRNA expression has been described in fetal membranes and placenta (Dennes et al., 1997). Here, NO has been attributed with roles in maintaining placental perfusion and myometrial quiescence during pregnancy through its actions as a vasodilator and smooth muscle relaxant (Dennes et al., 1997). Consistent with this, NO is produced by the decidua and myometrium throughout pregnancy, but with reduced NOS activity and myometrial responsiveness at term (Natuzzi et al., 1993; Sladek et al., 1993). Furthermore, inhibition of NO synthesis by administration of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) results in pre-eclamptic like symptoms and maternal hypertension in pregnant rats

(Buhimschi et al., 1996).

An apparently opposing function has also been proposed for NO in the tissue remodelling associated with cervical ripening (Chwalisz et al., 1997). Whilst decidual NO synthesis is downregulated at term, cervical NOS activity increases (Buhimschi et al., 1996) and local application of NO to the cervix in guinea pigs stimulates cervical ripening. A similar role may be envisaged in the inflammatory type response during menstruation, and additionally NO may influence menstrual blood loss through its vasodilatory action and inhibit platelet aggregation. It is also noteworthy that leukocytes within the mouse endometrium, including NK cells, mast cells and macrophages, have been identified as a source of NO, which may have important implications for leukocyte functioning at the materno-fetal interface' (Hunt et al., 1997).

These actions are frequently induced through stimulation of other local mediators. Many investigations have examined the interactions between NO and COX-2, due to their co-expression in many inflammatory situations (Vane et al., 1994; Dennes et al., 1997), with somewhat contradictory conclusions. It has, however, been proposed that NO acts to stimulate the inducible isoform of prostaglandin synthase, as demonstrated by both *in vitro* and *in vivo* studies (Salvemini, 1997), although the concentration of NO may determine the nature of the effect on COX-2 expression (Swierkosz et al., 1995). Further, as observed in most biological systems, a negative feedback loop is likely to exist, with stimulation of COX-2 by NO reported to be downregulated by PGE<sub>2</sub> mediated inhibition of iNOS (Tetsuka et al., 1994). A different situation exists for MCP-1, with two independent research groups demonstrating an inhibition of the chemokine by NO (Zeiher et al., 1995; Tsao et al., 1997). This, at least in part, explains the anti-inflammatory and protective role of NO in atherosclerosis, which is characterised by monocyte infiltrate. The significance of this in the endometrium is uncertain.

### 1.56 Prostaglandins

Another family of locally vasoactive substances are the prostaglandins (PGs) [For reviews see (Mitchell, 1992; Kelly, 1994)]. PGs are produced by *de novo* synthesis from fatty acid precursor arachidonic acid (AA) [see review, (Willis et al., 1994)], which is released from membrane bound phospholipids (phosphatidylethanolamine and phosphatidylcholine) by the activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) during cell signalling. Prostaglandin H endoperoxide synthase / cyclooxygenase (PGHS / COX)



catalyses the conversion to prostaglandins (Fraser et al, 1992). The conversion is rate limiting and consists of two reactions, both catalysed by COX. The first involves a double oxidation (cyclooxygenase) reaction producing unstable intermediate PGG<sub>2</sub>, which is rapidly converted to another biosynthetic intermediate PGH<sub>2</sub> by a peroxidase reaction. PGH<sub>2</sub> is rapidly modified by specific isomerases or synthases to produce specific prostanoids, the five most commonly produced being PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub>, PGI<sub>2</sub> (prostacyclin), and TXA<sub>2</sub> (thromboxane). PGE<sub>2</sub> and PGF<sub>2α</sub> are the most abundant prostaglandins in the human endometrium (Smith et al., 1988). PGE<sub>2</sub> is a potent vasodilator, and can induce stromal oedema in the presence of histamine or bradykinin. PGF<sub>2α</sub> has a contrasting role in stimulating vasoconstriction and cell activation.

Recently an inducible form of COX was discovered, COX-2 (PGHS-2) (see review, (Goppelt-Streube, 1995)), first identified as a phorbol ester inducible immediate early response gene in fibroblasts (TIS10) (Kubjuba et al., 1991; O'Banion et al., 1992). The two isoforms (COX-1 and COX-2), products of distinct genes, share 60% homology at the amino acid level, with noticeable differences in the regulatory regions (Kubjuba et al., 1991). COX-2 expression is dynamically regulated, with rapid transcription of the 4.1kb mRNA in response to many stimuli, including serum, growth factors (epidermal growth factor (EGF), PDGF), cytokines (IL-1, TNF-α), hormones (LH, hCG) (Han et al., 1996) and inflammatory mediators (phorbol ester, LPS) (Jacobs et al., 1994). It can be regulated both at the transcription level and by increasing the stability of the mRNA prior to translation. COX-2 displays many characteristics of an inducible gene, including multiple binding sites in the 5' untranslated region (e.g. TATA box) and rapid degradation of the mRNA, due to the presence of AUUUA target sequences for ribonucleases in the 3' untranslated end (Caput et al., 1986; Malter, 1989). It is rapidly degraded, thus conferring a sensitive response to environmental factors. Furthermore, an inhibition by glucocorticoids has been described (O'Banion et al., 1991). COX-2 could be very important in mediating local activation in a tissue and in pathological situations and thus is likely to be important for the mediation of acute inflammatory situations (Williams et al., 1973). COX-1, in comparison, is generally constitutively expressed, and has been proposed to fulfil the housekeeping role of prostaglandin production (Simmons et al., 1991; O'Banion et al., 1992). It is interesting that the isoforms of the COX enzyme are expressed at different subcellular compartments, with COX-2 primarily associated with the nuclear envelope, whilst COX-1 has been localised to the endoplasmic reticulum (Morita et al., 1995).

Well known for their roles in inflammatory responses mediated mainly through effects on the vasculature and immune system, the actions of prostaglandins in the uterus has been investigated in great detail. Initial work concentrated on the involvement of prostaglandins in mediating parturition, both by cervical ripening in an inflammatory type response, and induction of myometrial contractility (Calder et al., 1973; Calder, 1986). More recently the role of PGs and expression in the nonpregnant uterus has been studied, with a view to understanding normal and abnormal uterine function (Smith et al., 1981). Prostaglandins  $E_2$  and  $F_{2\alpha}$  ( $PGE_2$  and  $PGF_{2\alpha}$ ) are produced throughout the menstrual cycle, with well documented peaks in the mid-secretory phase and peri-menstrual period (Downie et al., 1974; Maathuis et al., 1978). An involvement of these vasoactive factors in menstruation was first realised by the detection of large amounts in menstrual fluid (Pickles et al., 1965). Elevated concentrations of vasodilatory prostaglandins,  $PGE_2$  and 6-keto- $PGF_{1\alpha}$  (metabolite of  $PGI_2$ ) are produced from endometrium of women suffering from menorrhagia (Smith et al., 1981; Cameron et al., 1987). PGs have been implicated in the changes in vascular tone associated with menses and further in the decidualisation of the uterine stroma in the mid-late secretory phase. The importance of the latter process has been discussed earlier with reference to prostaglandins role in implantation (section 1.14). Prostaglandins have been noted to accentuate the action of other mediators, for example,  $PGE$  has been observed to act synergistically with IL-8 in the chemotaxis of neutrophils into rabbit skin (Colditz, 1990) and a similar situation has been hypothesised in the uterus (Kelly et al., 1994). Prostaglandins could therefore play important roles both in the mediating and amplifying the inflammatory response which characterises menstruation.

The distribution of COX (before the identification of the second isoform) in the uterus has been studied by immunohistochemistry, with the majority of immunoreactivity in the epithelial glands (Rees et al., 1982). Additional expression of COX enzyme has been reported in the smooth muscle cells and endothelium of endometrial blood vessels (Rao et al., 1989). A preliminary investigation into the localisation of COX-2 found the enzyme present heterogeneously in the perivascular cells of small blood vessels (Kelly, 1994). The production of  $PGE$  in this position would support the proposed synergistic role of assisting neutrophil influx with IL-8, and also explain the vasodilatory action.

Interleukin-1 (IL-1) is an important modulator of PG production in the uterus, presumably through induction of COX-2. Decidual stromal cells have been demonstrated to produce COX-2 in response to IL-1 $\beta$  (Ishihara et al., 1992), and this

induction can be inhibited by dexamethasone and progesterone (Ishihara et al., 1995). This is supported by a downregulation of COX-2 by glucocorticoids in other cell types (O'Banion et al., 1991; Kubjuba et al., 1992). This finding reinforces the finding that prostaglandin release from endometrium is inhibited by progesterone. In the secretory phase, the capacity of the endometrium to produce prostaglandins is increased, with elevated levels of  $\text{PGF}_{2\alpha}$  reported (Downie et al., 1974; Abel et al., 1980; Singh et al., 1984). However, it appears that for the full potential to be realised, progesterone levels must fall, as they do premenstrually (Smith et al., 1988). In early pregnancy, basal production of PGs is generally low (Maathuis et al., 1978) and the tissue does not appear to retain the potential for trauma induced synthesis. Furthermore, in a culture system, prostaglandin production from proliferative and secretory endometrium is decreased by progesterone (Abel et al., 1980; Kelly et al., 1987).

Prostaglandins are important modulators of blood vessel tone, inflammation and the immune system (Williams et al., 1973; Kunkel, 1988). Tight regulation of prostaglandin activity is ensured both at the production level by cyclooxygenase expression and by their metabolism to the inactive 13-14-dihydro-15-keto forms by prostaglandin dehydrogenase (PGDH). PGDH is a progesterone responsive enzyme, as indicated by constantly elevated levels during pregnancy and in the early and mid luteal phases of the menstrual cycle (Casey et al., 1980). A peak in immunoreactive PGDH in the endometrium is observed in the secretory phase and during pregnancy, localised to the glandular epithelium. Administration of antigestogen RU486 in the early luteal phase (Cameron et al., 1997) and during early pregnancy acts to downregulate PGDH (Cheng et al., 1993b), resulting in increased levels of  $\text{PGE}_2$  around decidual blood vessels (Cheng et al., 1993a). Additionally, treatment of long term cultured endometrial cells with RU486 results in elevated  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  release in a dose dependant manner (Kelly et al., 1986). This helps to explain the increased sensitivity of the decidua to exogenous prostaglandins (stimulation of uterine contractions and bleeding), administered after mifepristone for the termination of pregnancy (Swahn et al., 1987; Brooks et al., 1990).

Prostaglandins are important agents in stimulating myometrial contraction during parturition. The PG output from amnion rises at the onset of spontaneous labour, and elevated levels of both  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$ , and their metabolites have been detected in amniotic fluid, maternal plasma and urine at this time. COX-2 has been reported to be the major isoenzyme expressed in amniochorion, and is upregulated during active labour (Slater et al., 1995). Additionally, COX-2 expression has been shown to be

higher than COX-1 in myometrium collected from pregnant women, with highest levels of COX-2 detected at term (Zuo et al., 1994) and during active labour (Slater et al., 1997). This has stimulated renewed interest into the blockade of prostaglandin production, using selective COX-2 non-steroidal anti-inflammatory agents to delay or prevent preterm labour (Sawdy et al., 1997). Although the amnion is a major producer of PGs, access of prostaglandins to the responsive myometrium is restricted by strong expression of the degradation enzyme PGDH by the intervening chorion. A further involvement for PGs in labour is in the ripening of the cervix (Calder, 1986).

PGs elicit a response by interaction with a family of G-protein coupled receptors possessing 7 putative transmembrane domains. To date, 5 classes of PG receptor have been isolated, DP, EP, FP, IP and TP (Coleman et al., 1994a). These have been classified by the specificity of binding to the subclasses of PGs, with greatest binding affinity of PGD to DP etc. Further complexity is achieved by the subdivision of at least two of the receptors, EP and TP, into subfamilies. Of particular interest are the EP receptors, comprising EP1, -2, -3 and -4. Differential physiological responses have been designated to individual receptors, most notably, EP1 and EP3 stimulate an excitatory response, whilst EP2 and the recently discovered EP4 elicit inhibitory responses and cause the vasodilatory action which characterises PGE<sub>2</sub> (Coleman et al., 1994b).

## **1.6 Prolactin**

Extra-pituitary prolactin (PRL) is a major product of the pregnant endometrium (Ben-Jonathon et al., 1996). Decidualised stromal cells have been conclusively shown to be the sole site of synthesis (Wu et al., 1995). Experimental studies have recognised that PRL also is produced by the non-pregnant endometrium, from the mid to late luteal phase until menses (Maslar et al., 1979). This corresponds to the initiation of decidualisation during which the stromal cells become pseudodecidualised (Wu et al., 1995), and *in vitro* decidualisation by stimulation of oestrogen primed proliferative endometrial cells with progesterone results in PRL production (Daly et al., 1983). Indeed, PRL expression is now commonly used as a marker for functional decidualisation. Although pituitary and endometrial PRL are identical in structure and function, expression is differentially regulated, which has been attributed to the presence of a distinct promoter region located ~6kb upstream of the pituitary promoter (DiMattia et al., 1990; Gellerson et al., 1994).



The significance of PRL expression in the endometrium is uncertain. It is believed that PRL primarily acts as an autocrine or paracrine rather than endocrine factor. A similar mechanism has been reported in immune cells, where PRL has a profound immunomodulatory effect (Draca, 1995). The majority of leukocytes ubiquitously express the prolactin receptor (PRL-R) (Pellegrini et al., 1992; Dardenne et al., 1994; Chambers et al., 1995; Wu et al., 1996). Stimulated T cells secrete PRL (Pellegrini et al., 1992), and *in vitro* PRL enhances the proliferation of leukocytes previously stimulated with mitogenic factors, including interleukin-2 (IL-2) and T and B cell mitogens (Clevenger et al., 1990). Furthermore, this proliferation is inhibited by exposure to anti-prolactin antibodies (Hartman et al., 1989). Prolactin, produced both by lymphocytes and from the pituitary, has been postulated as having a role in T and B cell competence and in the activation of T lymphocytes (Leite de Moraes et al., 1995). *In vivo* studies indicate an immunosuppressive action, such as the reduced immune response in lactating or PRL treated castrated rats (Dineen et al., 1972; Kelly et al., 1973).

The temporal expression of PRL in the endometrium suggests a role in the preparation for implantation and subsequent placentation. Decidualisation is essential for implantation to occur and the specialised immune environment within the decidua plays a major role in achieving a successful pregnancy. Coincidental with decidualisation is the accumulation of leukocytes in the endometrial stroma, the majority of which are the uterine specific large granular lymphocytes (LGLs) and macrophages (Bulmer, 1995; Loke et al., 1995a). PRL may be acting as an immunomodulatory agent in the endometrium, either by the stimulation of leukocyte proliferation or differentiation.

## 1.7 Hypothesis

The present study aims to further investigate the roles and actions of locally produced and acting inflammatory mediators in the endometrium. From previous research discussed in the introduction, a hypothesis of mediator interactions can be postulated (Figure 5).

A leukocyte accumulation is observed in the mid-late secretory phase of the normal menstrual cycle, corresponding to proposed vital roles in implantation, placentation and in the absence of pregnancy, in menstruation. Preliminary studies have reported the expression of the chemokine IL-8 in the non-pregnant and pregnant endometrium, localised to blood vessels. Prostaglandin production at a similar site has been identified, further verifying the proposed synergism in leukocyte recruitment. In addition, a downregulation of these local factors by the steroid hormone progesterone has been suggested.

Withdrawal of progesterone in the premenstrual period could have an effect on the local production of inflammatory mediators, by removal of transcriptional inhibition of chemokines, IL-8 and MCP-1, and activation of COX-2, whilst downregulating prostaglandin metabolism by PGDH. This would result in elevated levels of the chemokines and prostaglandins in the endometrium. Subsequently, chemokine directed migration of leukocytes into the endometrium could take place, enhanced by the vasodilatory action of PGE<sub>2</sub>. Endometrial leukocytes, once activated by chemokines, produce an array of cytokines, chemokines and prostaglandins, thus amplifying leukocyte recruitment and stromal oedema. Neutrophils and macrophages are, in addition, potential sources of MMPs or pro-MMP activating proteases, and thus may participate in ECM and tissue breakdown prior to menses.

The mechanism of appearance of the unique large granular lymphocyte population (CD56<sup>+</sup>CD16<sup>-</sup>) within the endometrium is unknown. The C-C chemokine MCP-1, along with family members MIP-1 $\alpha$  and RANTES, has been attributed with NK cell chemotactic activity and thus it is possible that a circulating precursor NK cell could be recruited. This is supported by the existence of a putative precursor cell type (CD56<sup>+</sup>CD16<sup>-</sup> agranular cells) which comprise around 1% of the circulating NK cells (Lanier, et al, 1986). A subsequent activation / differentiation to produce the LGL phenotype could then be proposed. A possible candidate for the differentiation is prolactin which in addition to having major immunoregulatory roles in peripheral immune system, is present in large quantities in the endometrium during

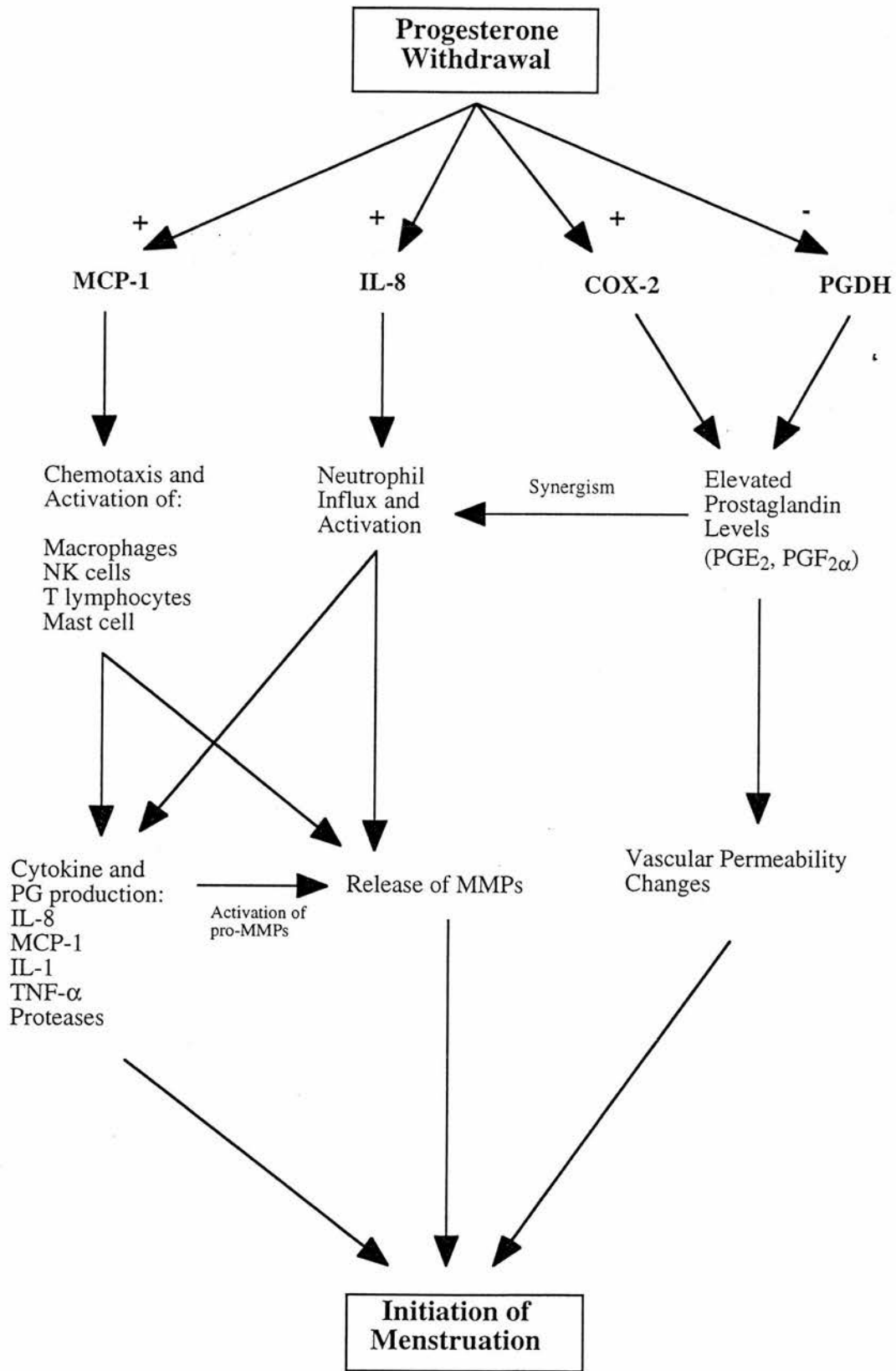


decidualisation and pregnancy. Furthermore, MCP-1 production is augmented by prolactin (Bowen et al, 1996). There are many other agents, detectable in the endometrium, which *in vitro* stimulate NK cell differentiation, including MCP-1 itself and other local factors such as IL-15, IL-12 and PGE.

The expression of all four local factors, IL-8, MCP-1, COX-2 and PRL, shall be examined in human endometrium, with particular emphasis on spatial and temporal expression patterns of these local mediators. Correlation with prevailing steroid hormone levels and perturbation of progesterone levels both *in vitro* and *in vivo* will help to clarify the complex regulation of local inflammatory mediators.

Increased knowledge of the role and regulation of locally acting mediators will greatly enhance our understanding of normal uterine function. This in turn has major implications for the development of novel contraceptive methods and treatments for pathological complaints including subfertility and dysfunctional menstruation.

**Figure 5**



## 1.8 Aims of Research Project

The aims of this research were to examine the involvement of locally acting factors in endometrial function, particularly focusing on the processes of implantation and menstruation. Specifically, the following questions were addressed:

1. Are the local factors, IL-8, MCP-1 and COX-2 detectable at the protein and mRNA level in the endometrium, throughout the menstrual cycle and in early pregnancy?
2. How do the spatial and temporal expression patterns of the above local mediators relate to their putative physiological roles?
4. Is there any evidence for steroidal control of these elements, particularly a progestogenic regulation?
5. Can the physiological pattern of expression be reproduced *in vivo* by clinical perturbation of steroid environment and in an *in vitro* culture system?
6. Does decidual derived prolactin have an immunomodulatory role in the uterus?
7. Do the above findings help to clarify the complex interactions of locally acting factors in the endometrium?

## **CHAPTER 2**

### **GENERAL MATERIALS AND METHODS**

## 2.1 Materials

The sources of all materials used for the experiments described in all chapters are presented below. These have been subdivided into experimental categories for ease of reference.

TISSUE COLLECTION MATERIALS	SOURCE
Neutral Buffered Formalin (NBF)	See appendix for recipe
Tissue-Tek O.C.T. Compound (Tissue Embedding Medium)	Tissue-Tek, Miles Inc., Slough, UK
Peel-A-way Disposal Embedding Moulds	Polysciences Inc., Warrington, USA
Nunc Cryotubes	Gibco, Paisley, UK
RPMI Culture Medium	Gibco, Paisley, UK
Isopentane	Merck Ltd. (BDH), Lutterworth, UK

IMMUNOHISTOCHEMISTRY	SOURCE
Histoclear	National Diagnostics, Atlanta, Georgia, USA
MCP-1 rabbit polyclonal antibody	Rabbit Polyclonal made "in house" , see text for sequence information
IL-8 rabbit polyclonal antibody	Rabbit Polyclonal made "in house" , see text for sequence information
COX-2 rabbit polyclonal antibody	PG-27, Oxford biomedical, Biogenesis, Poole, UK
Prolactin receptor rabbit polyclonal antibody	Kind gift from Dr P. Ingleton, University of Sheffield, Sheffield, UK
Phosphate Buffered Saline / Phosphate Buffered Saline with Tween PBS / PBS(T)	See appendix for recipes
Tris Buffered Saline / Tris Buffered Saline with Tween TBS / TBS(T)	See appendix for recipes
Hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> )	Merck Ltd. (BDH), Lutterworth, UK

<b>IMMUNOHISTOCHEMISTRY</b>	<b>SOURCE</b>
Normal goat / mouse / rabbit sera	Vectastain kits, Vector laboratories, Peterborough, UK
Biotinylated secondary antibodies (Goat anti-rabbit / Horse anti-mouse)	Vectastain kits, Vector laboratories, Peterborough, UK
Rabbit anti-mouse immunoglobulins	Z0259, DAKO, High Wycombe, U.K.
ABC-HRP and Elite complexes	Vectastain 4002 & Vectastain Elite PK-6101, Vectastain kits, Vector laboratories, Peterborough, UK
Alkaline phosphatase anti-alkaline phosphatase (APAAP)	Mouse monoclonal, D0651, DAKO, High Wycombe, U.K.
3, 3' -diaminobenzidine (DAB) substrate	Vector SK-4100, Vector laboratories, Peterborough, UK
Vector Red substrate	Vector laboratories, Peterborough, UK
Sodium citrate buffer	See appendix for recipe
Pertex Mounting Medium	Cellpath plc, Hemel Hempsted, UK
Harris's Haematoxylin	Pioneer Research Chemicals Ltd., Colchester, UK
Microscope Glass Slides	Merck Ltd. (BDH), Lutterworth, UK
Glass Coverslips	Merck Ltd. (BDH), Lutterworth, UK

<b>CELL CULTURE MATERIALS</b>	<b>SOURCE</b>
RPMI culture medium	Sigma-Aldrich Company Ltd., Poole, UK
Fetal Calf Serum (FCS)	Sigma-Aldrich Company Ltd., Poole, UK
Penicillin / Streptomycin	Sigma-Aldrich Company Ltd., Poole, UK
Gentamycin	Sigma-Aldrich Company Ltd., Poole, UK
Progesterone	Sigma-Aldrich Company Ltd., Poole, UK
Phorbol 12-Myristate 13-Acetate	Sigma-Aldrich Company Ltd., Poole, UK



<b>CELL CULTURE MATERIALS</b>	<b>SOURCE</b>
Ultraspec™ RNA Isolation System	Biotecx, Biogenesis, Poole, UK
Chloroform	Sigma-Aldrich Company Ltd., Poole, UK
24 well culture plates	Costar, Ltd. UK, High Wycombe, UK
Isopropanol	Sigma-Aldrich Company Ltd., Poole, UK

<b>REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)</b>	<b>SOURCE</b>
Avian Myeloblastosis Virus (AMV) Reverse Transcriptase	Promega Ltd, Southampton, UK
5 x Reverse Transcriptase buffer	Promega Ltd, Southampton, UK
PCR Nucleotide mix (premixed 10mM deoxynucleotide solution)	Boehringer Mannheim, Sussex, UK
Oligonucleotide Primers : GAP-DH	Oswell, Southampton, UK - for sequence data see table 3
Oligonucleotide Primers : IL-8	Oswell, Southampton, UK - for sequence data see table 3
Oligonucleotide Primers : MCP-1	Oswell, Southampton, UK - for sequence data see table 3
Oligonucleotide Primers : COX-2	Oswell, Southampton, UK - for sequence data see table 3
Oligonucleotide Primers : PRL-R	Genesis - for sequence data see table 3
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich Company Ltd., Poole, UK
Recombinant ribonuclease inhibitor (RNAsin)	Promega Ltd, Southampton, UK
AmpliTaq Polymerase	Perkin Elmer, Beaconsfield, UK
10 x PCR buffer	Perkin Elmer, Beaconsfield, UK
Magnesium chloride (MgCl <sub>2</sub> ; Molecular Grade)	Perkin Elmer, Beaconsfield, UK
Agarose (multipurpose)	Boehringer Mannheim, Sussex, UK

<b>REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)</b>	<b>SOURCE</b>
PCR Thermal Cyclers (Omnigene; Omni-E; Touchdown)	Hybaid Ltd., Middlesex, UK
Ethidium bromide	Sigma-Aldrich Company Ltd., Poole, UK
Molecular weight markers: PCR Marker (50-200bp)	Sigma-Aldrich Company Ltd., Poole, UK
DNA Molecular weight marker (72-1353bp)	Boehringer Mannheim, Sussex, UK
Gel loading buffer	See appendix for recipe
TBE buffer	See appendix for recipe

<b>ELISA MATERIALS</b>	<b>SOURCE</b>
Streptavidin coated microtitre plates (High Capacity)	Boehringer Mannheim, Sussex, UK
PBS-BSA	See appendix for recipe
Tris-EDTA (TE) buffer	See appendix for recipe
Denaturing solution	See appendix for recipe
5' Digoxigenin labelled probe: GAP-DH	Oswell, Southampton, UK - for sequence data see table 3
5' Digoxigenin labelled probe: IL-8	Oswell, Southampton, UK - for sequence data see table 3
5' Digoxigenin labelled probe: MCP-1	Oswell, Southampton, UK - for sequence data see table 3
5' Digoxigenin labelled probe: COX-2	Oswell, Southampton, UK - for sequence data see table 3
Easy Hyb Hybridisation buffer	Boehringer Mannheim, Sussex, UK
Anti-Digoxigenin Fab fragments, conjugated with peroxidase	Boehringer Mannheim, Sussex, UK
ELISA Wash buffer	See appendix for recipe

ELISA MATERIALS	SOURCE
ELISA buffer	See appendix for recipe
Peroxidase Substrate	See appendix for recipe
Sulphuric Acid (H <sub>2</sub> SO <sub>4</sub> )	Merck Ltd. (BDH), Lutterworth, UK

## **2.2 Methods**

### **2.21 Tissue Collection and Patient Details**

Normal endometrium from all stages of the menstrual cycle ( $n = 83$ ) was collected by Pipelle suction curette (Laboratoire CCD, Paris, France) from fertile women undergoing benign gynaecological procedures, including laparoscopic sterilisation and hysterectomy. All women reported normal regular menstrual cycles (18 - 34 days) and had not received any form of hormonal treatment for the 3 months preceding biopsy collection. Endometrial biopsies were dated from the patient's last menstrual period (LMP). Tissue sections were routinely stained with haematoxylin and eosin and examined by light microscopy to ensure that histological appearances were consistent with the date of LMP. In cases of uncertainty, the stage of cycle was confirmed by radioimmunoassay of serum concentrations of oestradiol and progesterone.

First trimester decidua ( $n = 33$ ) was collected away from the implantation site by careful curettage prior to termination of pregnancy (gestation 8 - 12 weeks) by vacuum aspiration. Decidua parietalis, without trophoblast invasion, was confirmed by cytokeratin immunostaining of tissue sections (see method in immunohistochemistry section 2.22).

Written informed consent was obtained prior to tissue collection and ethical approval was received from Lothian Research Ethics Committee (ref. no. 1702/94/6/1).

**Table 2** Table describing patient details, from whom endometrial samples were collected. No significant differences were detected in the cycle length described by women allocated to different stages of the menstrual cycle or early pregnancy. However, the age of patients consenting to collection of first trimester decidua was significantly less than that of women from whom non-pregnant endometrium was biopsied (\*  $p < 0.001$  compared to all non-pregnant groups). Additionally, significantly more patients were nulliparous in the pregnant group compared to all non-pregnant groups ( $p < 0.001$  - not shown in table).

Stage	Days of cycle	Number of samples	Cycle length Mean (range)	Age range. Mean (range)
Menstrual	d1 - 4	9	29 (28-31)	35.6 (25 - 47)
Early Proliferative	d5 - 7	10	26 (18-29)	34.2 (26 - 41)
Mid Proliferative	d8 - 10	10	27 (20-29)	35.6 (24 - 46)
Late Proliferative	d11 - 13	5	28 (25-32)	39.3 (33 - 46)
Periovulatory	d14	9	28 (21-32)	40.3 (31 - 49)
Early Secretory	d15 - 18	18	27 (21-31)	39.26 (30 - 47)
Mid Secretory	d19 - 24	11	28 (21-34)	38.6 (35 - 45)
Late Secretory	d25 - menses	11	29 (28-31)	38.9 (34 - 45)
First Trimester	8 - 12 weeks	33	28 (27-29)	23.3 (16 - 40) *
Decidua	gestation			

Tissue samples were divided into four pieces on a sterile surgical swab and were immediately placed into:

1. 10% Neutral Buffered Formalin (NBF) and fixed overnight at 4°C, then stored in 70% ethanol prior to routine paraffin embedding.
2. OCT embedding medium in a disposable plastic mould, snap frozen in iso-pentane precooled with dry ice and stored at -75°C.
3. Cryotube, snap frozen in iso-pentane precooled with dry ice, and stored at



-75°C prior to RNA extraction.

4. RPMI culture medium on ice, for tissue culture.

A venous blood sample was also collected at the time of biopsy from which serum was collected by centrifugation at 1,500g for 10 minutes. This was frozen at -20°C until measurement of steroid hormone concentration by radioimmunoassay, for confirmation of the stage of menstrual cycle (Yong et al., 1992).

## **2.22 Immunohistochemistry**

Immunohistochemical protocols for the detection of MCP-1, IL-8, COX-2 (Jones et al., 1997) and prolactin receptor (Jones et al., 1998) were optimised to determine the correct conditions for maximal specific staining. Methodology for the immunolocalisation of the chemokine IL-8 has previously been reported (Critchley et al., 1994). Suitable positive and negative controls for immunostaining were included in every run, to assess the specificity of immunoreactivity and ensure the comparability of individual staining runs.

### **IL-8**

Immunohistochemical localisation for IL-8 required the use of frozen sections (5µm in thickness), cut from the OCT embedded blocks. Immediately prior to staining, frozen sections were lightly fixed in 10% NBF for 10 minutes at room temperature. Sections were then washed in 0.1M Phosphate Buffered Saline (PBS) pH 7.4 - 7.6. Endogenous peroxidase activity was quenched by immersion of the slides in 3% Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) in distilled water for 5 minutes at room temperature. Following 10 minutes washing in PBS, diluted non-immune goat serum was applied to each slide and incubated for 20 minutes in a humidified chamber. The excess serum was carefully blotted off and 50µl of anti-IL-8 antibody, at a dilution of 1:500 in PBS, was applied. The IL-8 rabbit polyclonal antisera was raised in rabbits against a full length synthetic IL-8 peptide (72 amino acids), synthesised by fluorenylmethoxycarbonyl (Fmoc) chemistry. The antigen was administered in Freund's complete adjuvant (2mg/ml) with three boosts over 12 weeks using Freund's incomplete adjuvant. The cross reactivity's of the antibody were analysed by radioimmunoassay resulting in less than 0.25% cross reaction with interleukin's (IL) -1, -2, -6, and -10 and less than 0.3% against monocyte chemoattractant protein-1 (MCP-1). Coverslips were placed over the tissue sections to minimise antibody evaporation and were incubated for 60 minutes at 37°C. Following primary antibody

binding, an avidin biotin peroxidase detection system was utilised, involving the sequential application of biotinylated goat anti-rabbit IgG and a complex of avidin and biotin with horse radish peroxidase (ABC-HRP). The positive binding was then identified by the application of the peroxidase substrate 3, 3' -diaminobenzidine (DAB), which produces a brown stain representing the presence of IL-8 protein. Sections were then washed in distilled water and lightly counterstained with Harris's haematoxylin, a non-specific purple nuclear stain. Following washing in tap water the sections were dehydrated in ascending grades of ethanol and mounted from Xylene with Pertex mounting medium.

Frozen tonsil tissue sections were included as a positive control for IL-8 immunostaining procedures (Critchley et al., 1994) and were treated in an identical fashion. The presence of intense immunoreactivity surrounding blood vessels was indicative of specific immunoreactivity. To exclude the possibility of non-specific immunoreactivity, serial sections of representative slides were included and either non-immune rabbit immunoglobulins at matching concentrations or primary antibody preabsorbed with synthetic IL-8 peptide (100µg/ml) was substituted for the primary antibody.

### **MCP-1**

A similar protocol was used for MCP-1 immunolocalisation, with the exception of an overnight incubation period (17hrs  $\pm$  1 hr) with the primary antibody. MCP-1 rabbit polyclonal antibody was raised in rabbits against a chemically synthesised 77aa MCP-1 and used at a dilution of 1:400 in PBS. Identical positive and negative controls were used for MCP-1 as for IL-8. Primary antibody preabsorbed with MCP-1 synthetic peptide (100µg/ml) was utilised as a negative control. Further, western analysis of ovarian tissue for MCP-1 resulted in the detection of a band of the correct protein weight (10kDa) (data not shown).

### **COX-2**

Immunostaining for COX-2 was conducted on paraffin sections. These were dewaxed in for 5 minutes in Histoclear, and then rehydrated through descending grades of ethanol to distilled water. Prior to endogenous peroxidase blocking, a microwave antigen retrieval technique was utilised, whereby the sections were heated in sodium citrate buffer (pH 6.0) for 10 minutes at high power followed by a 20 minute incubation in the oven. A similar protocol as for MCP-1 was then continued, with the exception of 60 minute incubation periods with the primary

antibody, raised against COX-2 (PG27) diluted 1:250 at 37°C, and the secondary biotinylated goat anti-rabbit antibody and the tertiary avidin-biotin-peroxidase complex (ABC Elite), both at room temperature.

Human term fetal membranes were included as a positive control for COX-2 immunostaining, to ensure that the pattern of immunoreactivity achieved was in agreement with published data (Gibb et al., 1996). Serial sections of representative samples were treated with non-immunised rabbit immunoglobulins at a matching concentration to the primary antibody to serve as a negative control.

### **Prolactin Receptor (PRL-R)**

As for COX-2 immunostaining, paraffin sections were utilised for prolactin receptor immunohistochemistry. An antigen retrieval technique was not required. Sections were incubated with the primary antibody (rabbit polyclonal, raised against the highly conserved extracellular domain of the rat prolactin receptor) at a dilution of 1:50 overnight (17hrs±1hr) at 4°C. The biotinylated secondary goat anti-rabbit antibody and tertiary avidin-biotin-peroxidase complex were applied for 30 minutes each prior to substrate development with DAB. As for COX-2, human term fetal membranes were used as a positive control (Maaskant et al., 1996) and rabbit immunoglobulins diluted to 1:50 were substituted for the primary antibody on negative control sections.

### **Dual Immunohistochemistry**

To co-localise prolactin receptors with endometrial leukocyte subpopulations, dual immunohistochemistry was conducted. Paraffin sections were treated as for prolactin receptor single immunostain and then washed in TBS for 20 minutes. An immunolocalisation for Leukocyte Common Antigen (LCA - CD45) was then carried out by the application of the primary antibody at a dilution of 1:50 in TBS for 60 minutes at 37°C. An alkaline phosphatase detection system was utilised, involving the application of the secondary antibody rabbit anti-mouse immunoglobulins at a dilution of 1:25 for 60 minutes, followed by the tertiary antibody alkaline phosphatase anti alkaline phosphatase (APAAP) diluted 1:50 in TBS for 60 minutes. The phosphatase substrate, Vector red was then applied for 30 minutes, producing a bright pink precipitate over the leukocytes isolated by the CD45 antibody. Sections were counterstained with haematoxylin and examined to compare localisation of CD45 positive and prolactin receptor positive cells. Serial sections were treated with either prolactin receptor or CD45 antibody followed by or

after treatment with mouse IgG / rabbit IgG, to assess the specificity of the single immunostain. A negative control exposed to mouse IgG and rabbit IgG was also included.

## **Cytokeratin**

Immunohistochemistry for cytokeratin was performed on first trimester decidua biopsies to confirm the identify of the tissue as decidua parietalis opposed to basalis, on the basis of whether trophoblast invasion had occurred. Paraffin or frozen sections were utilised and were exposed to anti-cytokeratin monoclonal antibody for 1 hour at 37°C at a dilution of 1 in 60 . No pretreatments were required for antigen exposure. Cytokeratin positive cells were identified by the application of biotinylated horse anti-mouse immunoglobulins followed by avidin-biotin-peroxidase complex (ABC-HRP) for 30 minutes at room temperature. DAB was utilised as the substrate producing a brown precipitate over the antigen-antibody complex. In all tissues immunoreactivity was detected in both surface and glandular epithelium. The presence of immunostaining outwith these structures was indicative of trophoblast invasion, and thus these tissues were not included in any further studies. Proliferative endometrium with prominent epithelial glands were included to act as a positive control for the immunostain. Mouse immunoglobulins at a matching concentration was substituted for the primary antibody to act as a negative control.

### **2.23 Scoring and Analysis of Immunostaining**

A semi-quantitative scoring system was employed for assessment of intensity and localisation of immunoreactivity in the entire tissue section, utilising a 4 point scoring system where 0 = no immunostaining, 1 = faint immunostaining, 2 = strong immunostaining and 3 = very intense immunostaining. Large differences were observed in staining intensity, and all tissue sections were randomly coded and thus examined blind to the stage of the cycle by either one or two observers. We are not aware of any image analysis software which would enable accurate assessment of diffuse cytoplasmic immunostaining, such as is produced from chemokine and COX-2 immunohistochemistry. Statistical analysis was conducted on a Apple Macintosh Computer using the statistical package Statview version 4.5. Analysis of Variance (ANOVA) with Fisher's PLSD post hoc was utilised to evaluate whether or not there were significant differences in the expression of the mediators.



## 2.24 RNA Extraction from Whole Tissue

Tissue from which RNA was to be extracted was placed directly into a sterile eppendorf containing 1ml of Ultraspec™ RNA Isolation Medium. Tissue snap frozen in cryotubes was semi-thawed and treated identically. The tissue was homogenised and placed at 4°C for 10 minutes to allow complete dissociation of nucleoprotein complexes. 200µl of chloroform was added to each tube and mixed thoroughly by shaking for 15 seconds. Following another 10 minutes on ice, the tubes were centrifuged at 12,000g for 20 minutes at 4°C in a Biofuge refrigerated centrifuge. The homogenate separates into an upper aqueous phase containing RNA, and the interface and organic phases containing proteins and DNA. To precipitate the RNA, the aqueous phase was aspirated into an equal volume of iso-propanol, mixed and placed at -20°C overnight (17hrs ± 1hr). The precipitated RNA was collected in a white pellet by centrifuging the eppendorf at 12,000g for 15 minutes at 4°C. The pellet was washed twice to remove contaminating phenol and chloroform with 75% ethanol, by vortexing briefly and centrifuging at 7,500g for 5 minutes. The pellet was then air dried and resuspended in 30-50µl of diethylpyrocarbonate (DEPC) treated water.

An approximation of RNA yield was attained by measuring the absorbance at 260nm by spectrophotometry. RNA concentration was calculated using the following equation:

$$\text{RNA Concentration } \mu\text{g}/\mu\text{l} = \text{Absorbance at 260nm} \times 40 \times \text{dilution factor}$$

where an optical density (OD) of 1 is equal to an RNA concentration of 40 µg/µl

The purity of the extracted RNA was assessed by the ratio of 260nm:280nm, where a pure RNA sample produces a value of 1.8 - 2.0. The RNA samples were stored at -75°C until further use.

## 2.25 Non-Competitive Semi-Quantitative RT-PCR

RT-PCR was utilised to determine the expression levels of the chemokines, COX-2 and prolactin receptor in the endometrium. Insufficient quantities of RNA were extracted for analysis by northern hybridisation or ribonuclease protection assay, but the gross amplification and high degree of sensitivity involved in the RT-PCR technique enables the visualisation of product from very low numbers of mRNA transcripts. To enable quantitation of the RT-PCR products and assess viability of



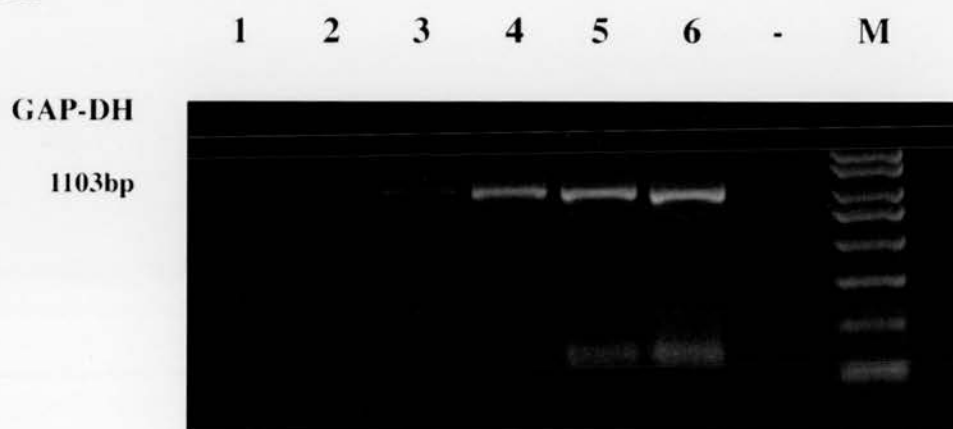
the RNA samples, the expression levels of the housekeeping gene Glyceraldehydephosphate dehydrogenase (GAP-DH) (Murphy et al., 1990; Belin, 1996) were assessed by RT-PCR from all RNA samples. Antisense oligonucleotide primers utilised for GAP-DH and mediator amplification by RT-PCR were 5' labelled with biotin, thus generating biotinylated RT-PCR products. This allowed specific detection and quantification of the products by enzyme linked immunoabsorbant assay (ELISA) using a streptavidin coated microtitre plate.

The concentration of GAP-DH RT-PCR products were determined using this method. This was related to the concentration of initial RNA template loaded into the reaction, by the production of a standard curve of RNA template (x) plotted against PCR product (y). A linear relationship was achieved, indicating that the PCR yield was proportional to the amount of RNA starting material. This situation can only be achieved when the PCR reaction is in the exponential phase of amplification, and thus is not limited by the presence of insufficient reaction components (see figures 6, 7, 8). This was ensured by optimisation of all RT-PCR reactions.

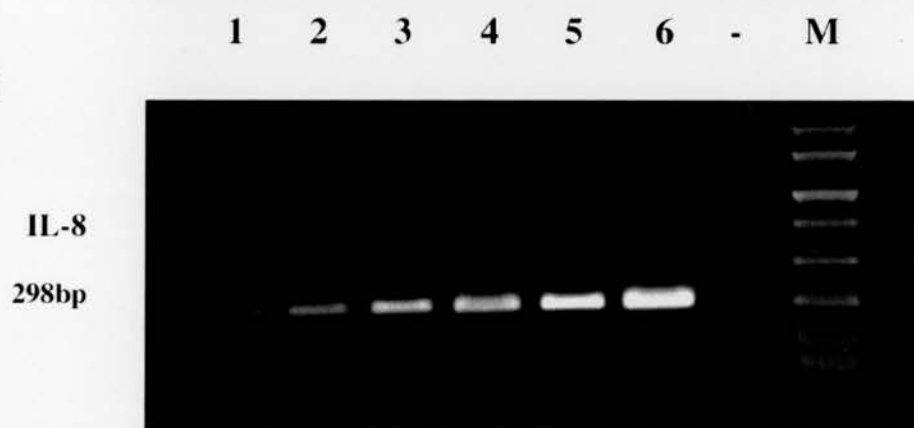
The protocols of thermal cycling was optimised to produce a single band of the correct molecular weight for each target mRNA. This involved varying the temperature at which the oligonucleotide primer anneals to the template, the length of time allowed for the denaturing, annealing and extension stages and the number of amplification cycles used. The number of cycles at which the amplification is exponential was selected by conducting the PCR reaction at a range of cycle numbers (e.g. from 20 - 35 cycles) with a chosen amount of RNA template (60ng) (see figures 6, 7, 8). Products were analysed by gel electrophoresis and ELISA. The relationship between PCR yield and cycle number was examined graphically, and the number of cycles to be used in future studies was selected from the exponential region of the plot.

**Figure 6** Optimisation of RT-PCRs for GAP-DH, IL-8 and COX-2. A representative sample was amplified by RT-PCR for a range of amplification cycles, to determine when the amplification reaction was exponential in nature. For GAP-DH, 1 = 20 cycles, 2 = 23, 3 = 26, 4 = 29, 5 = 32 and 6 = 35. For IL-8 and COX-2, 1 = 25 cycles, 2 = 28, 3 = 31, 4 = 34, 5 = 37, 6 = 40 (IL-8 only). A negative control is included which was subjected to the maximum number of cycles and products were compared with molecular weight marker (M).

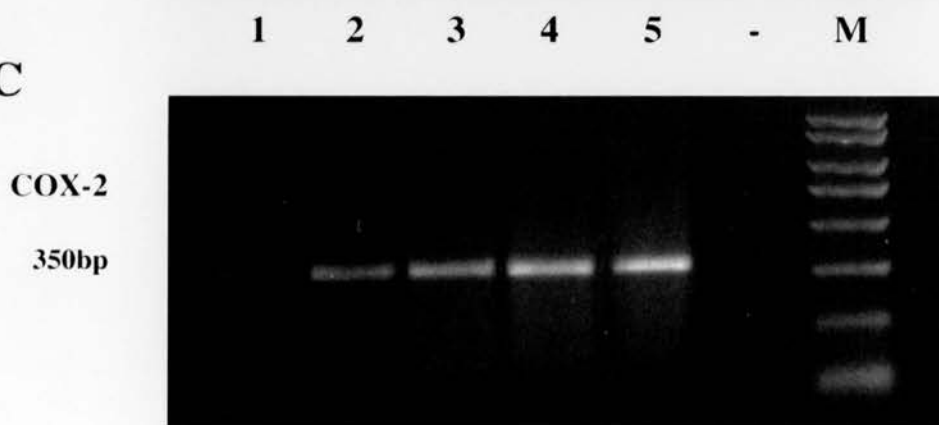
**A**



**B**



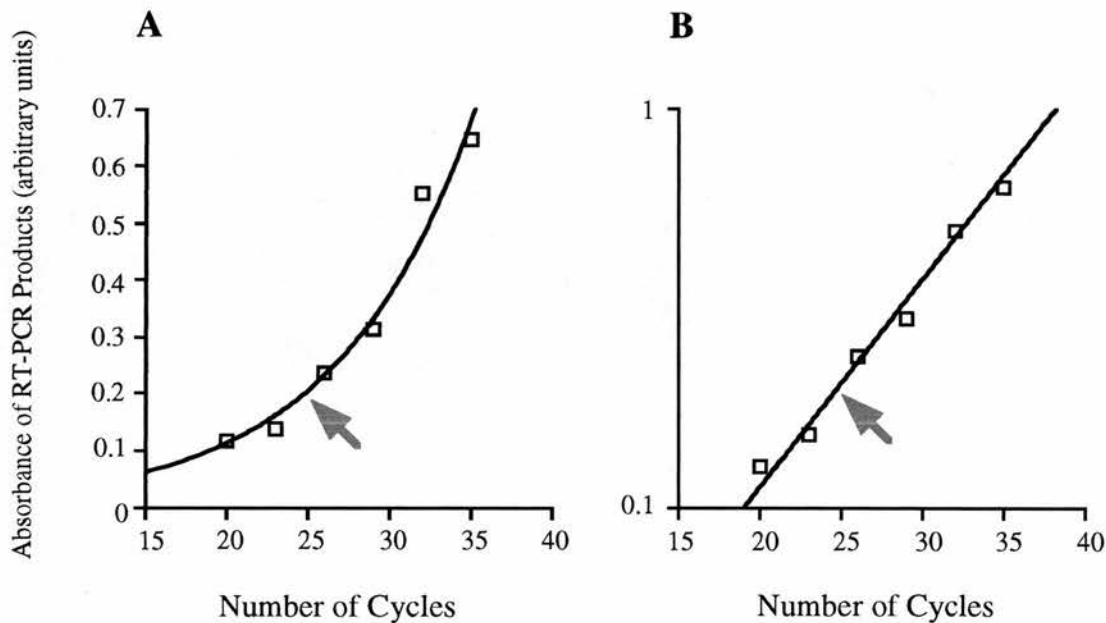
**C**



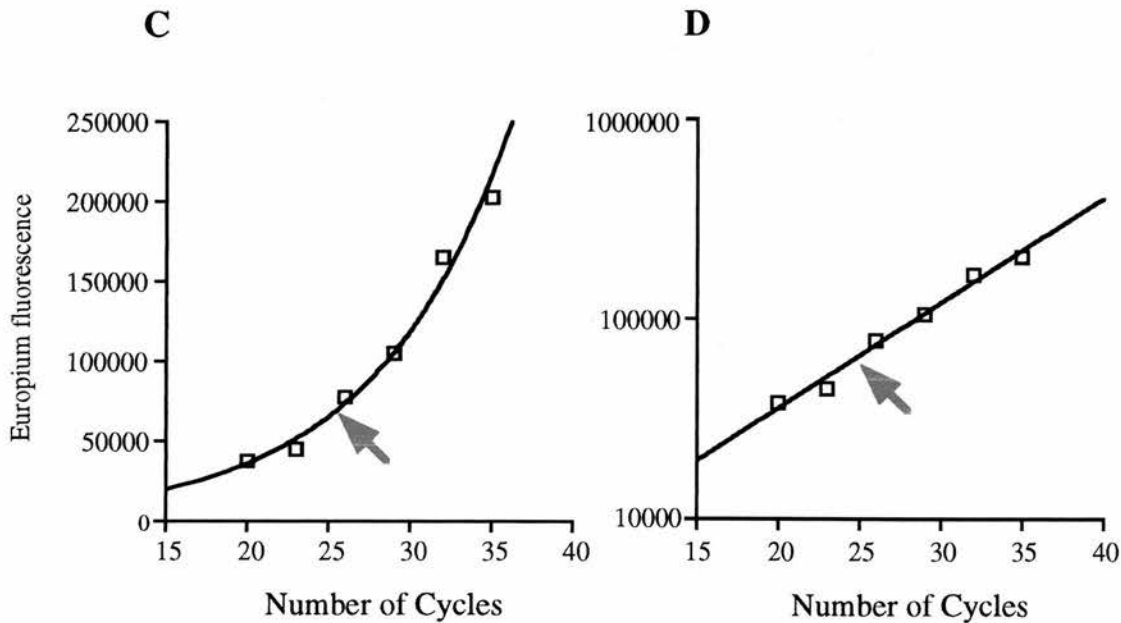
**Figure 7** Optimisation of GAP-DH RT-PCR. The product yield of RT-PCR reactions shown overleaf were measured by ELISA and expressed graphically, using different detection systems: (**A & B**) an antibody directed against digoxigenin-conjugated with peroxidase, and (**C & D**) a fluorescence (europium) labelled probe. Figure **A** shows the absorbance of RT-PCR product plotted against cycle number. This produces an exponential relationship, confirmed in figure **B**, where absorbance of RT-PCR product is expressed on a logarithmic scale, producing a straight line. The arrow demonstrates the number of cycles chosen for all subsequent reactions, when the reaction is in the exponential phase of amplification. Figures **C & D** illustrate the same findings, but an alternative detection system is utilised. This further validates the use of 26 cycles for GAP-DH RT-PCR reactions.

**Figure 7**

$$y = 0.010 * 10^{0.052x}$$



$$y = 3122.884 * 10^{0.053x}$$

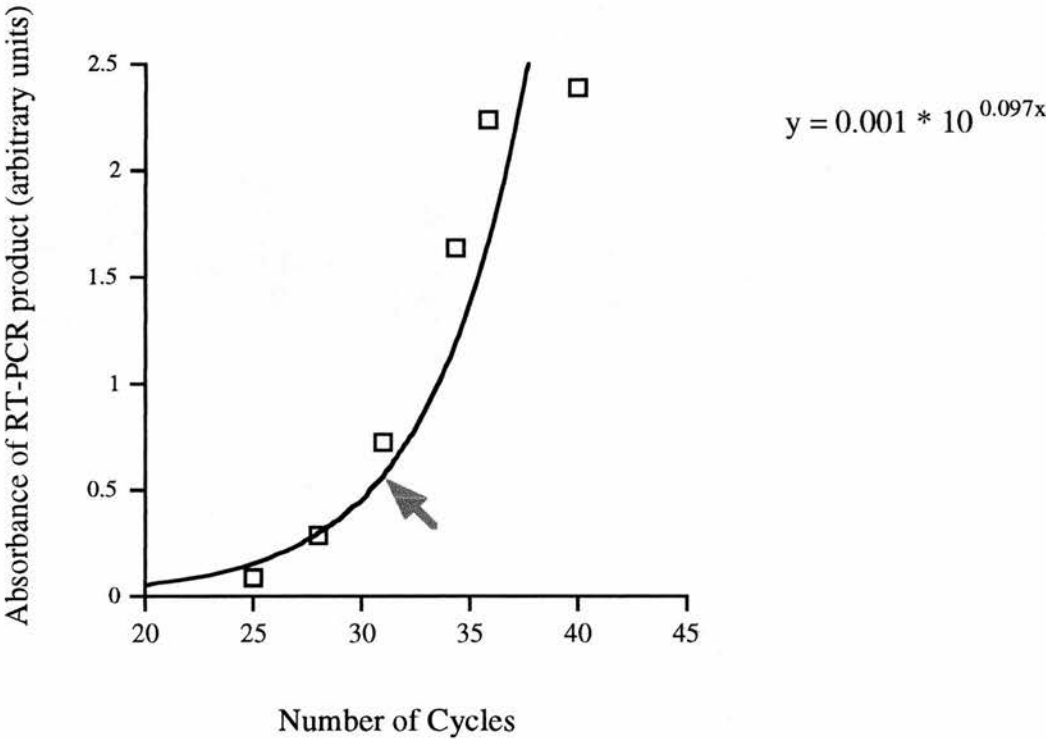


**Figure 8** Optimisation of IL-8 and COX-2 RT-PCRs. As for GAP-DH, product yield was measured by ELISA, adopting the anti-digoxigenin-peroxidase method. Figure **A** demonstrates the absorbance of IL-8 RT-PCR product, using an uncultured sample, plotted against the number of amplification cycles. Although at 40 cycles, the reaction is becoming limited by reaction components, at 32 cycles (indicated by the arrow), the reaction is in the exponential phase of amplification. For COX-2 (**B**), the reaction also appears to be limiting at 40 cycles for a cultured sample, but is very much exponential at 27 cycles (arrow).

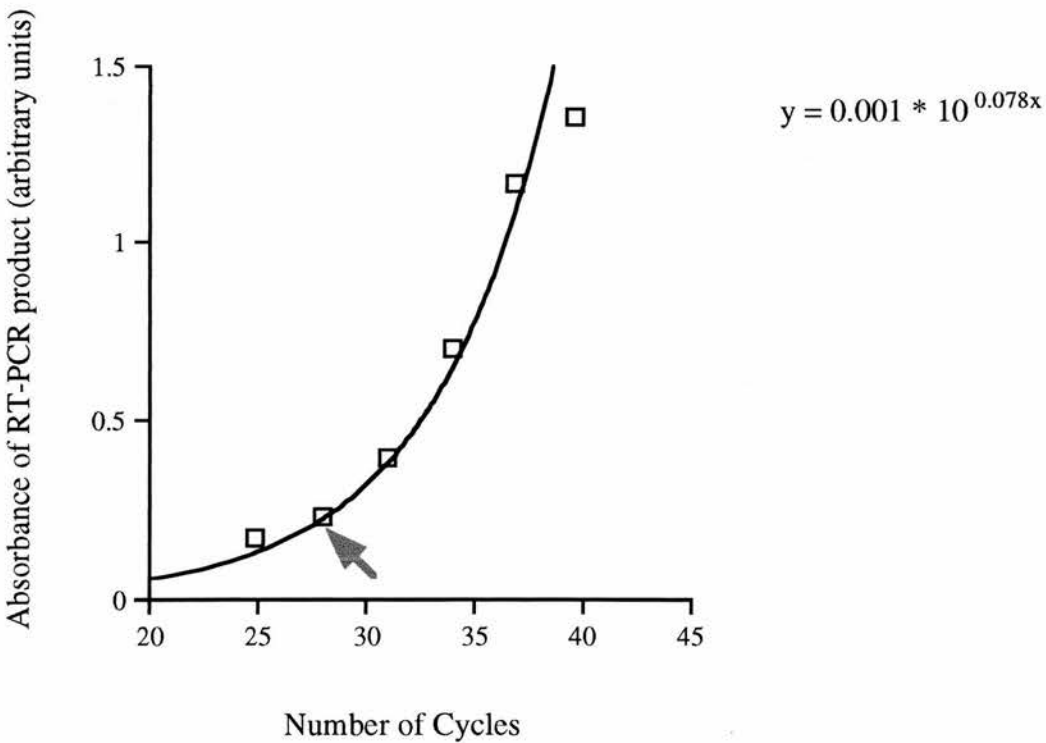


**Figure 8**

**A** IL-8



**B** COX-2



The aim of this study was to ensure that all RNA samples to be analysed for IL-8, MCP-1 and COX-2 mRNA expression were of equivalent concentration. Taking into account the proposed direct relationship between RNA loading into the RT-PCR and the product yield, this could be achieved when the concentrations of PCR product were comparable, as assessed by gel electrophoresis and ELISA. Thereafter, equal concentrations of sample RNA were analysed for the expression levels of mRNAs of interest, and the GAPDH product yield was measured once again by gel electrophoresis and ELISA.

## **Controls**

A number of measures were taken to ensure that all amplification was specific. A positive or quality control (QC, RNA extracted from a menstrual phase endometrial sample) was included in every RT-PCR. A negative control was achieved by the omission of RNA from the RT-PCR reaction. The possibility of amplification of genomic DNA contaminating RNA samples was excluded either by DNase treatment of RNA samples prior to analysis by RT-PCR, amplification of RNA not reverse transcribed by PCR or where possible by the use of oligonucleotide primers which span intronic regions of the gene. For all RT-PCR protocols these precautions were taken, to ensure that the product yield was solely due to amplification of cDNA generated from RNA template during the RT step.

## **GAP-DH RT-PCR**

To enable the concentration of sample RNA to be calculated, a standard curve was generated in each RT-PCR. This was created by the inclusion of 5 standards, diluted from a single RNA standard, in the GAP-DH RT-PCR. A decidual RNA sample was selected for use as a standard for all GAP-DH RT-PCR reactions, as a more accurate assessment of RNA concentration by spectrophotometry could be obtained due to the large quantity. A range of standard template concentrations between 15.7ng and 250ng were loaded into an RT-PCR, producing a standard curve of increasing product concentration (y) with increasing RNA template concentration (x). A similar curve was loaded into every RT-PCR reaction to reduce the inherent variability of this technique.

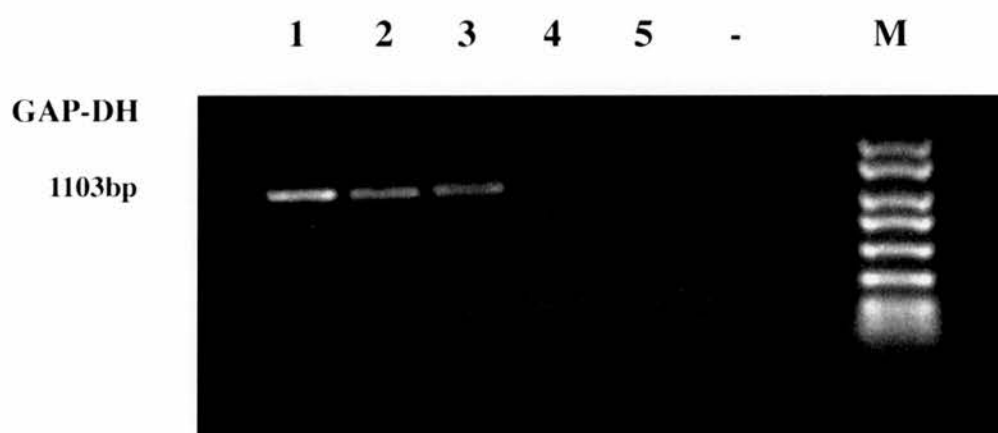
Gene specific cDNA was generated from equal volumes of the RNA samples to be analysed using AMV reverse transcriptase and the 5' biotinylated GAP-DH antisense oligonucleotide primer (see table 3, for detailed protocols see Appendix II). A master

mix of reaction components was made up for each reverse transcription run, so that each sample tube contained an identical reaction mix. The tubes were overlaid with a drop of RNase and DNase free mineral oil to prevent evaporation of the reaction components during heating. The eppendorfs were then centrifuged for 10 seconds to collect all reaction components under the mineral oil, placed in the thermal cycler (Hybaid Touchdown) and heated to 42°C for 45 minutes.

Following reverse transcription (RT) , the tubes were placed on ice and 30µl of PCR reaction mix containing 2.5 U AmpliTaq Polymerase was added to the total volume of RT products (20µl). A 1103bp fragment of the GAP-DH cDNA was selectively amplified by inclusion of the sense oligonucleotide primer (see table 3) and the same 5' biotinylated antisense oligonucleotide prime used for the RT step. The samples were subsequently subjected to 26 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes. A final extension of 10 minutes at 72°C was then allowed.

The PCR products were initially analysed by electrophoretic separation on a 1% agarose gel containing ethidium bromide. A molecular weight marker was included to allow size comparison with the samples. The gel was examined and photographed on a UV transilluminator.

**Figure 9** Standard curve for GAP-DH RT-PCR. A single RNA standard, at a range of RNA concentrations (1 = 250ng, 2 = 125ng, 3 = 62.5ng, 4 = 31.25ng, 5 = 15.7ng, - = negative control) was reverse transcribed and amplified, in every GAP-DH RT-PCR reaction, alongside samples to be analysed. A signal of 1103bp, by reference to a molecular weight marker (M), was detected for all samples, with decreasing intensity with decreasing amount of RNA loaded into the reaction.



## 2.26 GAP-DH ELISA

All RT-PCR products were diluted 1:10 in Tris-EDTA buffer and stored at  $-75^{\circ}\text{C}$  until assayed. 10 $\mu\text{l}$  of each product was pipetted into a high capacity 96 well microtitre plate coated with streptavidin, and a further dilution of 1:10 was achieved by the addition of 90 $\mu\text{l}$  PBS-BSA (0.1%). Specific GAP-DH amplicon binding via biotin-streptavidin was allowed by incubation of the plate at room temperature on a plate shaker for 20 minutes. Denaturing solution (50 $\mu\text{l}$ ) was added to ensure all DNA was single stranded. Following a 20 minute incubation with shaking, the contents of the well were discarded and specifically bound PCR products were detected by hybridisation, at  $50^{\circ}\text{C}$  for 1 hour, with a 5' digoxigenin labelled oligonucleotide probe (29 bp) (see table 3) complementary to the mid portion of the GAP-DH amplicon. Non-specifically bound probe was eliminated by repeated washing in specially formulated wash buffer. The probe - PCR product complex was detected by incubation with a polyclonal antibody raised against digoxigenin, conjugated with horse radish peroxidase (anti-DIG-POD Fab fragments) at a dilution of 1:1000 in ELISA buffer, for 45 minutes at room temperature with shaking. Following washing, the plate was developed with a peroxidase substrate for between 5 - 10 minutes. The reaction was then quenched with the addition of 2N  $\text{H}_2\text{SO}_4$ , and the absorbance read at a wavelength of 450nm (see figure 13).

Absorbances for product yield from standards and samples were found to be comparable with the signal intensity observed by gel electrophoresis. A standard curve was plotted of amount of RNA template loaded into the RT-PCR (x) against absorbance of standard RT-PCR product (y) (see figures 9 and 10). This generated the equation  $y = ax + b$ , which could subsequently be used to calculate sample RNA loading from their product yield. The RT-PCR and ELISA were subsequently repeated with corrected amounts of RNA template (60ng), to ensure that all RNA loading was equal. When all samples produced a measurement of 60ng (+/- 10ng), RT-PCRs for the mediators (IL-8, MCP-1 and COX-2) were conducted. All RT-PCR programs and ELISA protocols were similar to that described for GAP-DH, with the differences detailed below for each mediator.

### Europium ELISA

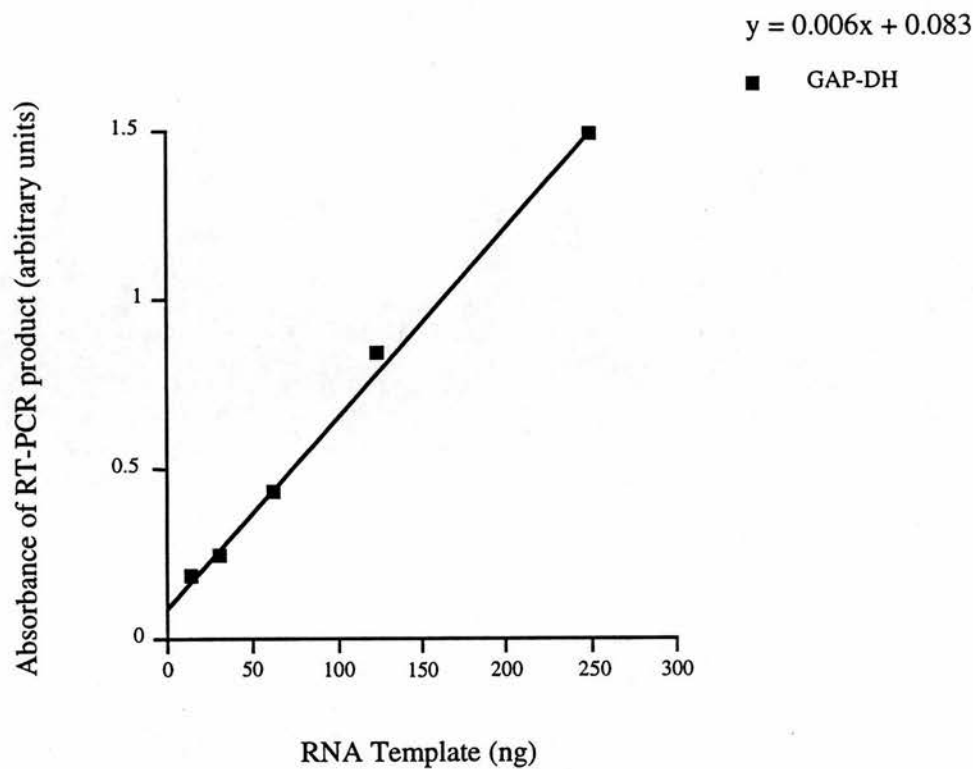
Detection of the probe amplicon complex using an antibody-enzyme system introduces the potential for further variability. To explore the validity of the optimised technique, fluorescent detection via direct europium labelling of the GAP-



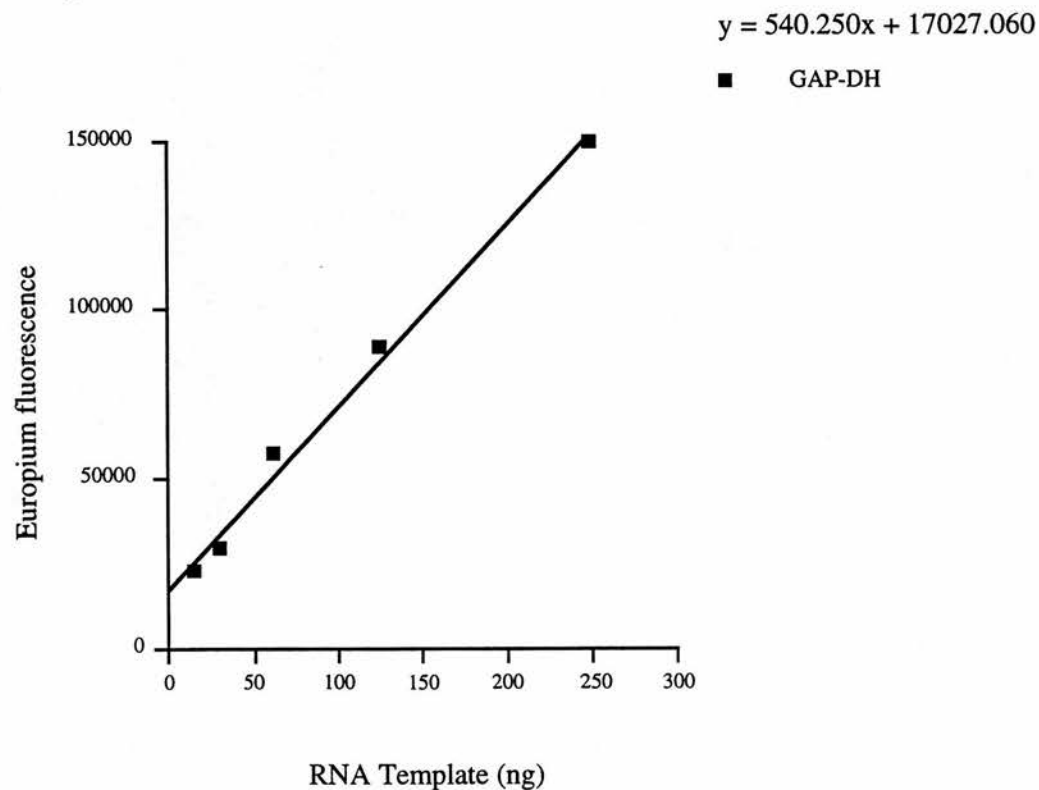
DH probe, was employed to enable comparison of the two different methods of ELISA. The advantage of the fluorescence detection system is the fact that fewer steps are involved in the detection process as the probe is directly labelled with europium, and thus fewer limitations are imposed. A representative GAP-DH standard curve was assayed using both methods simultaneously. Samples were diluted 1:200 in PBS-BSA, and 100µl was loaded into each well of a streptavidin coated microtitre plate, and incubated for 20 minutes with shaking. After denaturation of the PCR products, 100 µl of GAP-DH probe, labelled with Europium (1:1) was added and incubated at 50°C for 2 hours. Excess probe was removed by repeated washing and 200µl of fluorescence enhancing solution was pipetted into each well. After 20 minutes at room temperature, the fluorescence emitted was counted by a fluorimeter, and readings were plotted against the amount of RNA template. The results, presented in figure 10, correlate well, thus reinforcing the antibody-peroxidase detection system for ELISA as a suitable method of quantitation for RT-PCR.

**Figure 10** Standard curve for GAP-DH RT-PCR. Following amplification of standards for GAP-DH, product yield was measured by ELISA. Two methods were initially used for GAP-DH ELISA, using either (A) an antibody directed against digoxigenin-conjugated to peroxidase, or (B) a direct fluorescent labelled probe. A linear relationship was achieved, as the PCR was stopped in the exponential phase of amplification and thus the product yield is directly related to the amount of starting material. The antibody-peroxidase detection system was used thereafter and the RNA loading of samples was calculated from their product yield using the equation generated.

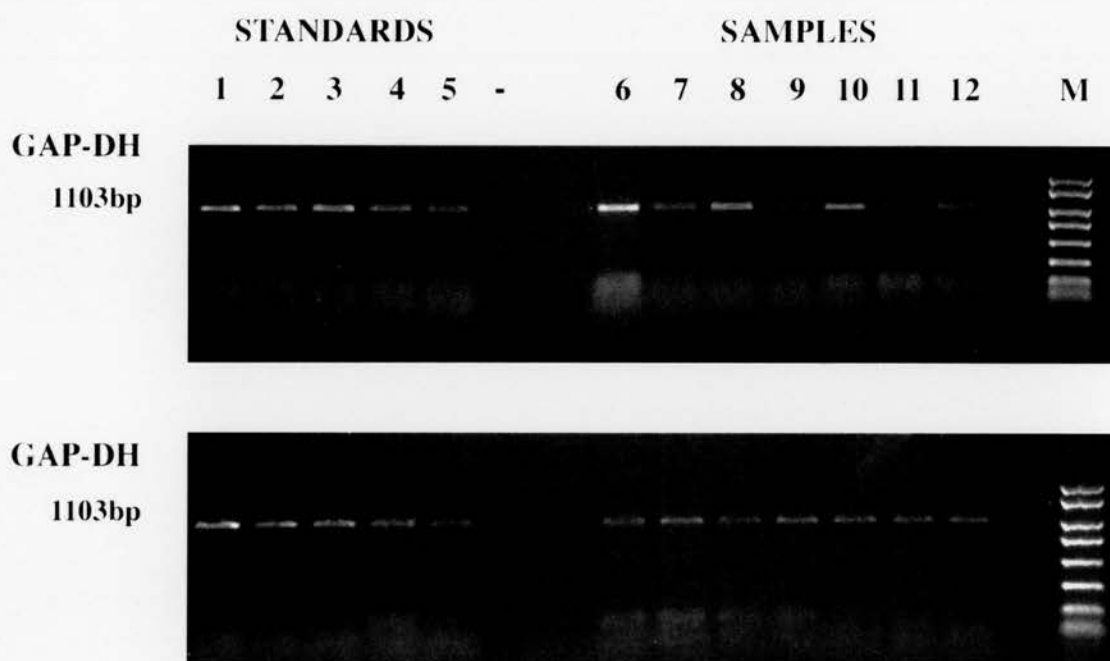
**A**     **Figure 10**



**B**



**Figure 11** The validity of this technique, for analysing RNA concentrations and adjusting them appropriately to ensure all samples are loaded equally (60ng), is illustrated in the composite opposite. The upper panel shows the products obtained from the first GAP-DH RT-PCR conducted on standards 1 - 5 (250ng - 15.7ng), and selected samples (6 - 12). For the samples, signals of variable intensity were obtained, indicating the uneven loading of the RT-PCR. The lower panel shows the same samples after adjustment. Again a standard curve (1 - 5) was included in the RT-PCR run, and samples yielded products of equal intensity when analysed by gel electrophoresis, and were all in the range of 60ng (+/- 10ng) after analysis by ELISA.



### **Figures 12 & 13**

The figures on the following two pages diagrammatically demonstrate the steps involved in the RT-PCR and ELISA techniques.



**Figure 12**

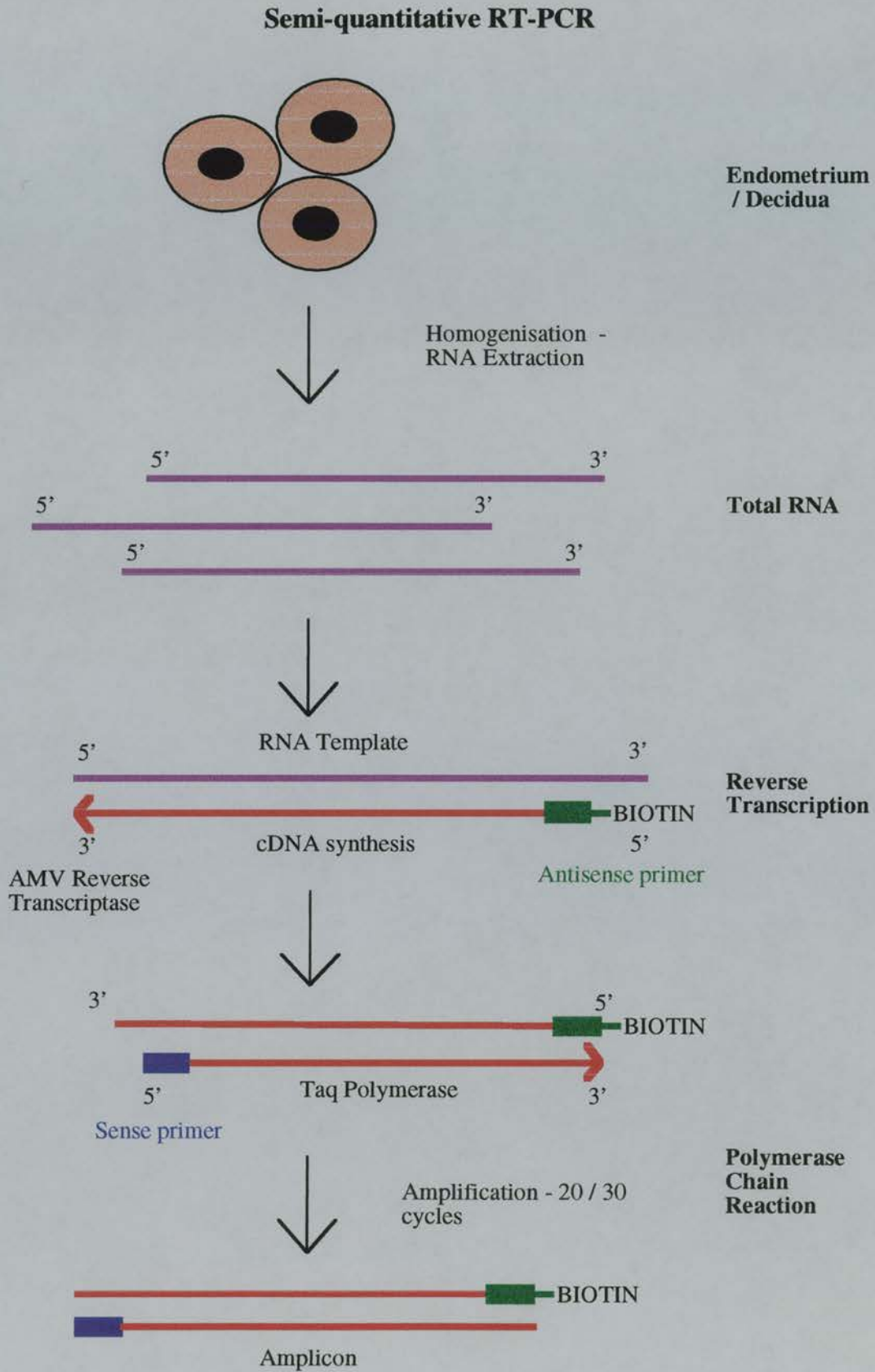
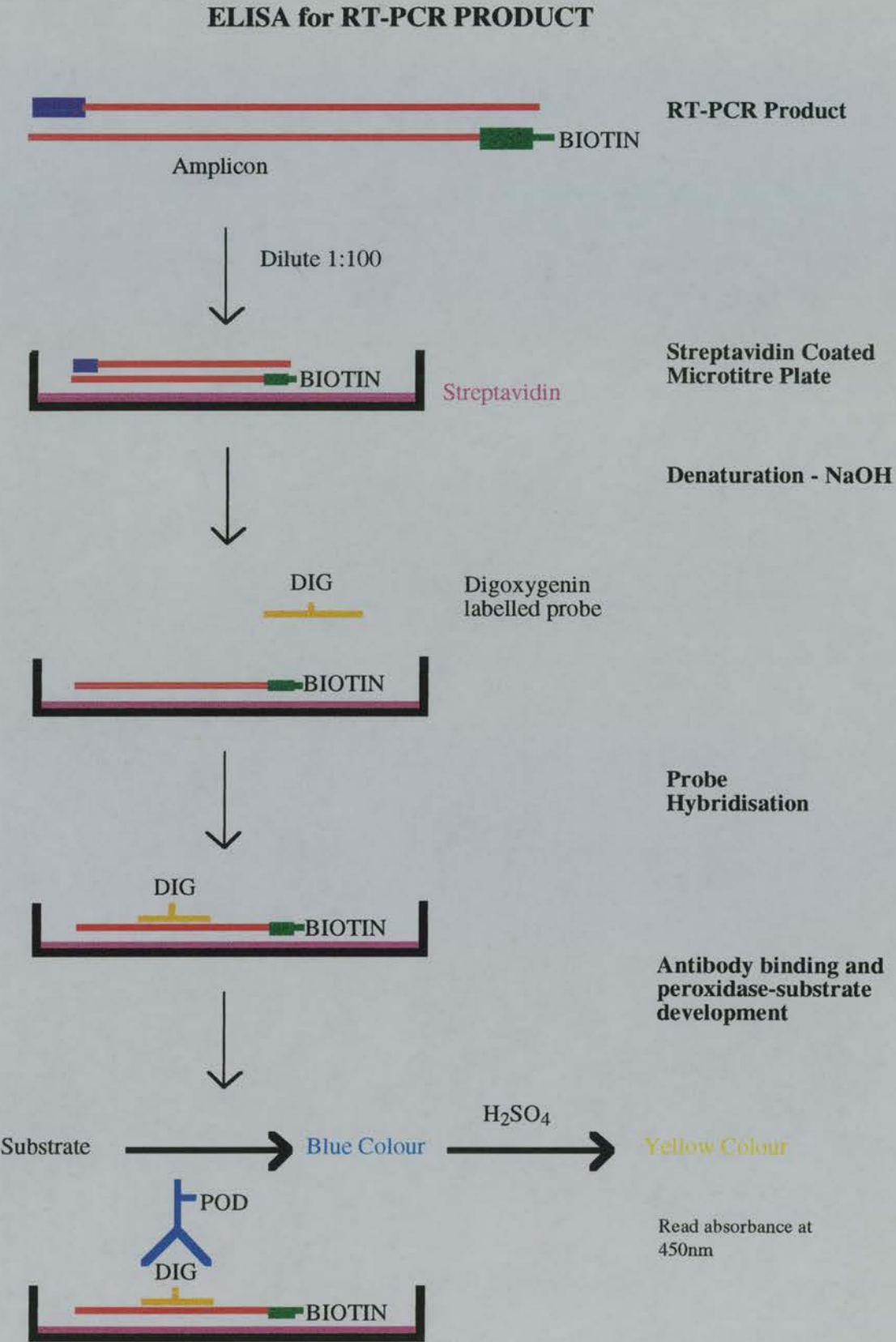


Figure 13



## **Mediator RT-PCR and ELISA**

The non-competitive method utilised in this study does not allow calculation of the exact number of mRNA transcripts of IL-8, MCP-1 or COX-2. Instead variations between samples collected at different times points throughout the menstrual cycle, or different culture treatments can be detected. It was not possible to include a standard curve of known mediator mRNA concentration as for GAP-DH, but to allow standardisation between individual RT-PCRs and ELISAs, a quality control was included in each procedure. The quality control utilised (QC) was a menstrual RNA sample, which from preliminary investigations proved to contain high numbers of transcripts for IL-8, MCP-1 and COX-2. Data obtained from measurement of RT-PCR product yield by ELISA has been presented as a ratio of sample signal': QC signal unless otherwise stated.

### Table 3

Table describing the sequences of all oligonucleotide primers utilised for RT-PCR, and ELISA probes complementary to the mid portion of the amplified product. The product size obtained for each RT-PCR is also provided. The optimised number of cycles, when the PCR was in the exponential phase of amplification, are in the final column. For IL-8, MCP-1 and COX-2 two different cycle numbers are given, as following stimulation of the tissue by culture (Chapter 5), these mediators were expressed at such high levels that it was impossible to measure mRNA levels quantitatively using the same PCR program as for unstimulated samples. In each case, the number of cycles used for unstimulated (uncultured) samples is given first, followed by the number required for detection of mediators in stimulated samples.

**Table 3**

	Oligonucleotides	Sequence 5' - 3'	Product size (bp)	Cycle number
GAP-DH	Antisense (5' Biotin)	CATGTGGGCCATGAGGTCCACCAC	1103	26
	Sense	TGAAGGTCGGAGTCAACGGATTTGGT		
	Internal probe (5' Digoxigenin)	CGTCATGGGTGTGAACCATGAGAAGTATG		
IL-8	Antisense (5' Biotin)	TGAATTCTCAGCCCTCTTCAAAAACCTTCTC	298	32 / 23
	Sense	ACTTCCAAGCTGGCCGTGGCTCTCTTGGCA		
	Internal probe (5' Digoxigenin)	GTGTGAAGGTGCAGTTTTGCCAAGGAGTGC		
MCP-1	Antisense (5' Biotin)	CGGAGTTTGGGTTTGCTTGTCC	210	32 / 23
	Sense	AATCAATGCCCCAGTCACCTGC		
	Internal probe (5' Digoxigenin)	GCTGTGATCTTCAAGACCATTTGTGGCC		
COX-2	Antisense (5' Biotin)	AGATCATCTCTGCCTGAGTATCTT	350	35 / 27
	Sense	TTCAAATGAGATTGTGGGAAAATTGCT		
	Internal probe (5' Digoxigenin)	GCTTTCACCAACGGGCTGGGCCATGGG		
PRL-R	Antisense	GCAGGTCACCATGCTATAGCCCTT	650	30
	Sense	GCAGATGGAGGACTTCCTACCAATTA		

### **IL-8 RT-PCR and ELISA**

The selective amplification of IL-8 was conducted using a pair of designed oligonucleotide primers to produce a 298bp fragment. RNA samples (60ng) were subjected to RT using an identical program as for GAP-DH (for detailed RT-PCR protocols see Appendix II). The generated cDNA was subsequently amplified by cycles of 94°C for 1 minute, 63°C for 1 minute and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. RNA extracted from uncultured samples were amplified for 32 cycles. The level of expression of IL-8 following a culture period were many fold those from uncultured tissue samples, necessitating the use of a much shorter cycling program (23 cycles). Therefore, limited comparisons between IL-8 expression levels in cultured and uncultured endometrial and decidual could be made.

### **MCP-1 RT-PCR and ELISA**

Oligonucleotide primer pairs were designed to amplify a 210bp fragment of MCP-1 mRNA. These spanned intronic sequences to act as a control for the possibility of genomic DNA contamination. A thermal cycling program was optimised to produce a single band corresponding to the region of MCP-1 cDNA selected, comprised of 32 cycles (uncultured biopsies) or 23 cycles (cultured biopsies) of 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute. A final extension at 72°C for 10 minutes was then allowed.

### **COX-2 RT-PCR and ELISA**

A 350bp amplicon was generated using the specific oligonucleotide primers for COX-2 (Hla et al., 1992). RT products were amplified by heating to 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute. Uncultured endometrial and decidual biopsies required 35 cycles, whilst cultured biopsies were subjected to 27 cycles of amplification. Once more, a final extension for 10 minutes at 72°C was allowed.

### **PRL-R**

Oligonucleotide primers complementary to the extracellular domain of the prolactin receptor flanking base pairs 154 - 798 were used to amplify by RT-PCR a 650bp product. The primers chosen spanned intronic sequences to ensure against the possibility of genomic DNA contamination. All samples analysed were uncultured



and were amplified for 30 cycles of 94°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute, followed by 10 minutes at 72°C.

### RT-PCR & ELISA Variability

The reproducibility and validity of this technique was investigated, and the variability of the RT-PCR, and intra- and inter-assay variations were calculated. All were determined to be less than 7%, based on variability between a minimum of 6 samples.

**Table 4**

Amplicon	RT-PCR Variability (PCR variability)	ELISA Intra-assay Variability	ELISA Inter-assay Variability
GAP-DH	6.52% (1.9%)	1.74%	2.62%
IL-8	4.91% (3.62%)	2.88%	4.23%
MCP-1	3.81% (1.47%)	3.37%	6.89%
COX-2	3.77% (3.23%)	2.11%	2.84%

## **CHAPTER 3**

### **LOCALISATION AND TEMPORAL EXPRESSION OF CHEMOKINES AND CYCLOOXYGENASE-2 IN HUMAN ENDOMETRIUM**

### 3.1 Introduction

The marked increase in endometrial leukocytes which coincides with the processes of implantation and menstruation strongly support a role in the modulation of these events (Loke et al., 1995a). Although *in situ* proliferation of leukocytes does occur in the endometrium (Pace et al., 1989; Tabibzadeh, 1990; King et al., 1991), infiltration of peripheral circulating leukocytes into the endometrial stroma is likely to contribute to their accumulation in the late secretory or premenstrual phase of the normal menstrual cycle (Lea et al., 1991; Marzusch et al., 1993). Identification of specific leukocyte chemoattractants in the endometrium would reinforce this hypothesis.

Interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) are two such chemotactic agents (Baggiolini et al., 1994; Baggiolini et al., 1997). IL-8, a C-X-C chemokine, selectively recruits neutrophils and T lymphocytes and triggers their subsequent activation upon tissue entry. Conversely, MCP-1 (C-C chemokine) has no effect on neutrophil chemotaxis, but instead is a potent chemoattractant and activator of monocytes, T lymphocytes, natural killer cells (NK), basophils and eosinophils. As neutrophils, macrophages and the NK-like LGLs constitute the majority of uterine lymphoid cells (Starkey et al., 1991), the investigation into temporal and spatial expression of these chemokines is of great interest.

IL-8 has previously been detected in the endometrium, localised to a perivascular position in endometrium obtained from both non-pregnant and pregnant women (Critchley et al., 1994). This preliminary study failed to identify any changes in expression level throughout the menstrual cycle. Further investigation has focused on modulation of IL-8 expression in human endometrial cells in culture (Arici et al., 1993; Arici et al., 1996). No definitive research into the temporal expression of this potent stimulator of neutrophil chemotaxis and angiogenesis in the endometrium has to date been conducted. MCP-1 production by ectopic endometrium biopsied from endometriotic lesions has been reported (Akoum et al., 1995), and has been related to the elevated numbers of activated macrophages, thought to contribute to the pathogenesis of this condition. MCP-1 gene expression by human endometrium and decidua was investigated, but again no changes directly relating to stage of cycle or pregnancy were detected (Arici et al., 1995).

The involvement of prostaglandins (PGs) in the regulation of normal endometrial function has long been recognised (Baird et al., 1996). An increase in PG production

by the endometrium has been detected in the mid-late secretory phase and during menses (Downie et al., 1974; Maathuis et al., 1978). PGE<sub>2</sub> is particularly implicated in the increased vasculature permeability which is a prerequisite for decidualisation (Kennedy et al., 1981) and therefore also implantation (Kennedy, 1986). Although a specific role for prostaglandins during menstruation has not been established, these vasoactive substances are presumed to have an integral role in the vasculature changes that characterise the perimenstrual and menstrual periods. Cyclooxygenase-2 (COX-2) is believed to be the main isoform responsible for the production of prostaglandins in situations of acute inflammation, due to its rapid induction by pro-inflammatory mediators and short half life (Simmons et al., 1991; Seibert et al., 1994). The processes of implantation and menstruation in many ways resemble an inflammatory response (Kelly et al., 1994). This study investigates the participation of COX-2 in the production of prostaglandins in the endometrium.

The present study has investigated the localisation and temporal expression of the chemokines IL-8 and MCP-1 and also COX-2 in normal endometrium throughout the menstrual cycle and in early pregnancy.

### 3.2 Materials and Methods

Endometrial (n = 50) and first trimester decidual biopsies (n = 24) were examined for immunolocalisation of the chemokines and COX-2 (table 5). All tissue samples were processed in OCT frozen and paraffin blocks (section 2.21). A further 20 endometrial samples and 5 decidual samples were examined for mediator expression by RT-PCR. Total RNA was extracted as described earlier (section 2.24) from tissue frozen in cryotubes.

**Table 5**

Endometrial and decidual samples utilised. Stage of menstrual cycle at time of biopsy collection.

STAGE OF CYCLE	DAYS FROM LMP	NUMBER OF BIOPSIES
Menstrual	d1-4	6
Early Proliferative	d5-7	4
Mid Proliferative	d8-10	4
Late Proliferative	d11-13	5
Ovulatory	d14	5
Early Secretory	d15-18	10
Mid Secretory	d19-24	9
Late Secretory	d25-mens	7
First Trimester Decidua	8-10 weeks amenorrhoea	24

#### Immunohistochemistry

Immunohistochemistry for IL-8, MCP-1 and COX-2 was conducted to detect and localise the mediators in normal endometrium at all stages of the menstrual cycle and in early pregnancy, utilising methods described in General Materials and Methods (section 2.22).

Immunostaining was examined by light microscopy and staining intensity and localisation was evaluated by semi-quantitative methods as described earlier (section 2.23).

### **Semi-quantitative RT-PCR**

Endometrial and decidual RNA samples were subjected to RT-PCR and ELISA for GAP-DH to determine their concentration, relative to a standard curve plotted of varying RNA template (x) against the absorbance of RT-PCR product (y). The RT-PCR was repeated with 60ng of each RNA sample as a template as verification of equal loading. The samples were amplified for IL-8, MCP-1 and COX-2, using protocols as described in general materials and methods (sections 2.25 and 2.26). RT-PCR product yield was measured by ELISA, and expressed as a ratio of sample signal : quality control (QC) signal. Gel electrophoresis was also conducted to illustrate the changes in mediator RNA expression levels.

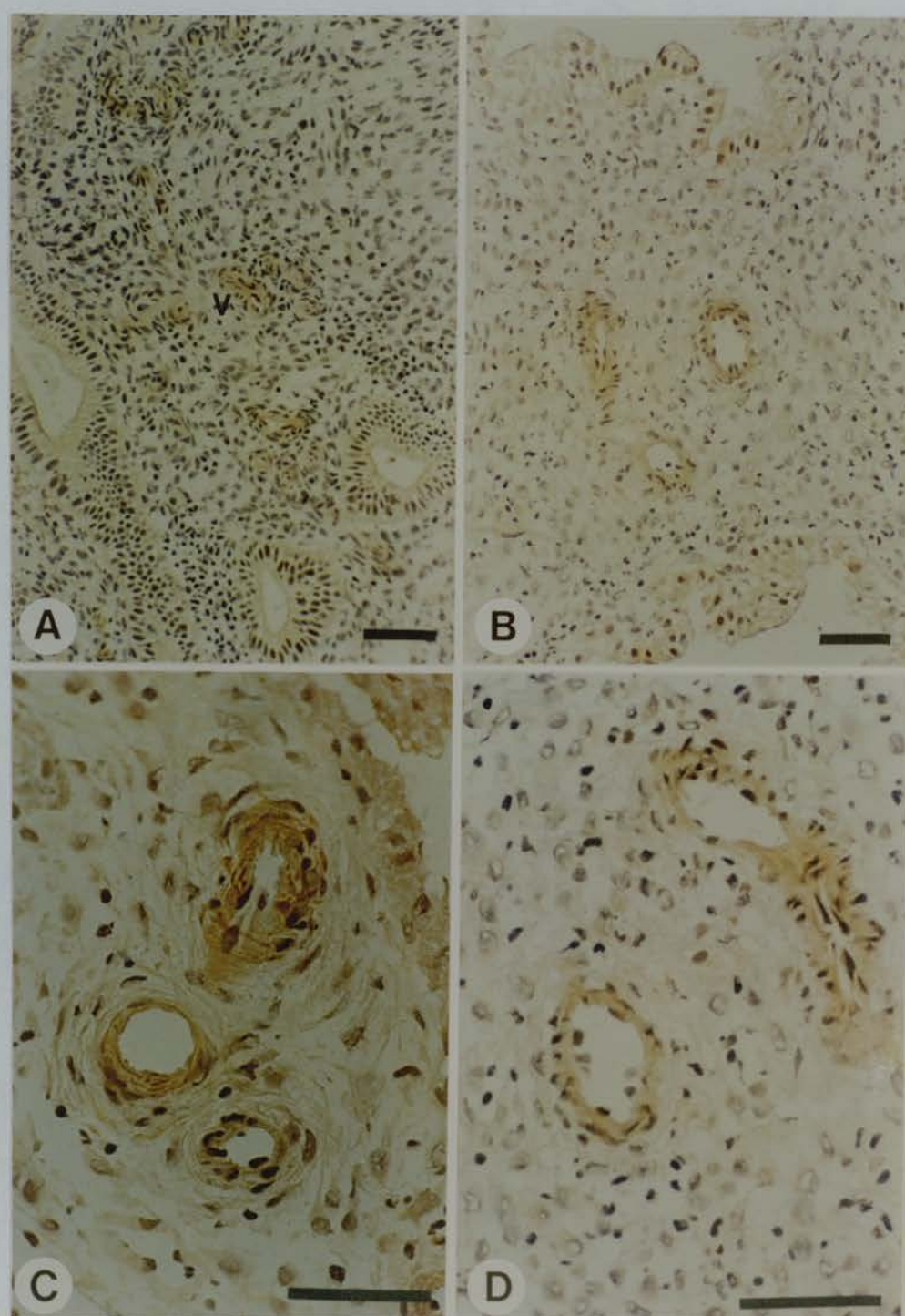


### 3.3 Results

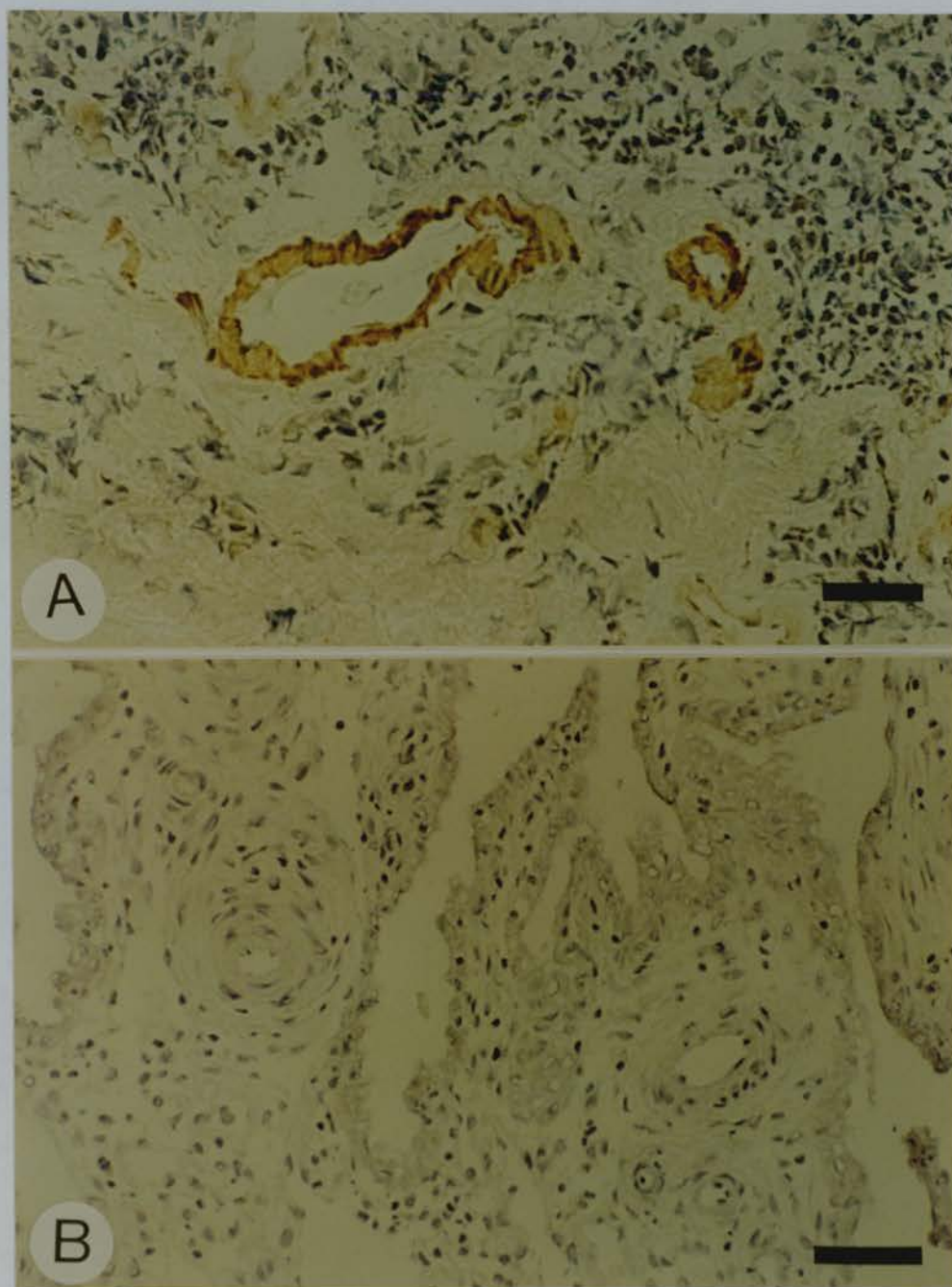
The local mediators, MCP-1, IL-8 and COX-2 were detected by immunohistochemistry in samples of endometrium from non-pregnant and pregnant women. Immunostaining was primarily present in the perivascular cells around all blood vessel types in the endometrial stroma for MCP-1 and IL-8, although weaker immunoreactivity was identified in the glandular epithelium for both chemokines (figures 14 - 17). Additional staining was detected for MCP-1 in a subpopulation of stromal cells in some decidual biopsies (figure 17). Intense immunoreactivity for COX-2 was detected in the epithelial glands and perivascular cells (figures 18 and 19). In the menstrual phase and in some of the decidual sections examined, a degree of stromal staining was observed.

Tonsil sections included as a positive control for MCP-1 and IL-8 immunostaining also exhibited perivascular immunostaining. Immunoreactivity for COX-2 in third trimester fetal membranes was detected primarily in the amniotic epithelium and chorion laeve trophoblast, with slight immunostaining in the attached decidual cells, in agreement with published data (Gibb et al., 1996). Antibody preabsorbed with the appropriate peptide or non-immune rabbit IgG applied to serial tissue sections to act as a negative control, resulted in an absence of immunoreactivity.

**Figure 14** Immunohistochemical localisation of IL-8 in human endometrium. IL-8 was localised to the perivascular cells in pregnant and non-pregnant endometrium, with distinct variations throughout the menstrual cycle. Faint immunoreactivity is apparent in (A) periovulatory and (B) early secretory phase endometrium around small blood vessels (v). (C) Increased intensity of perivascular IL-8 immunoreactivity was detected in late secretory endometrium, whilst in first trimester decidua (D), immunostaining was generally less pronounced. Scale bars = 50µm.



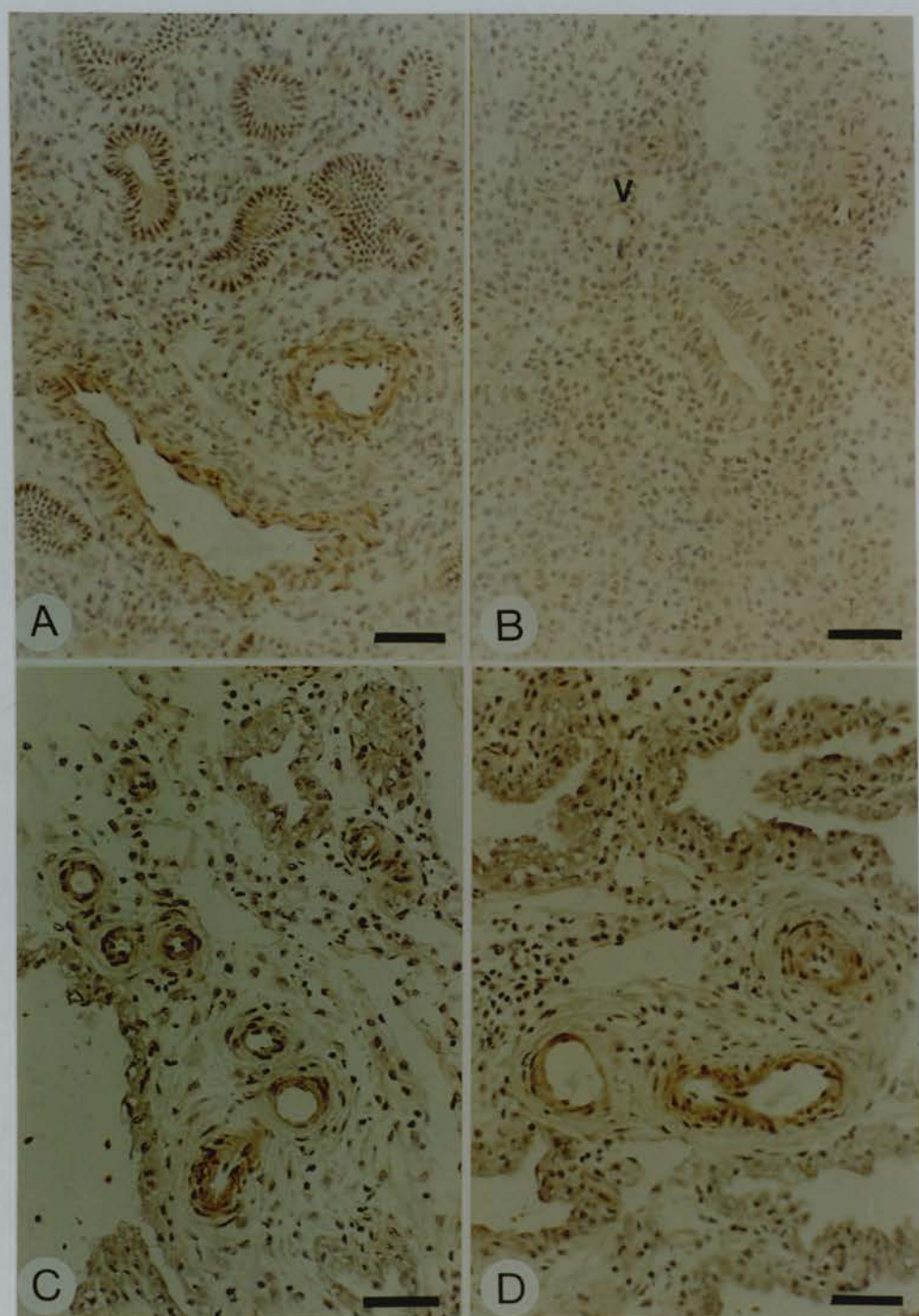
**Figure 15** Positive and negative controls for IL-8 immunohistochemistry. The upper photomicrograph demonstrates tonsil tissue section immunostained for IL-8. As in the endometrium, immunoreactivity was restricted to the vasculature. To ensure the specificity of the immunostaining, the antibody was preabsorbed with synthetic IL-8 (100µg/ml) for 24 hours, resulting in an absence of staining.



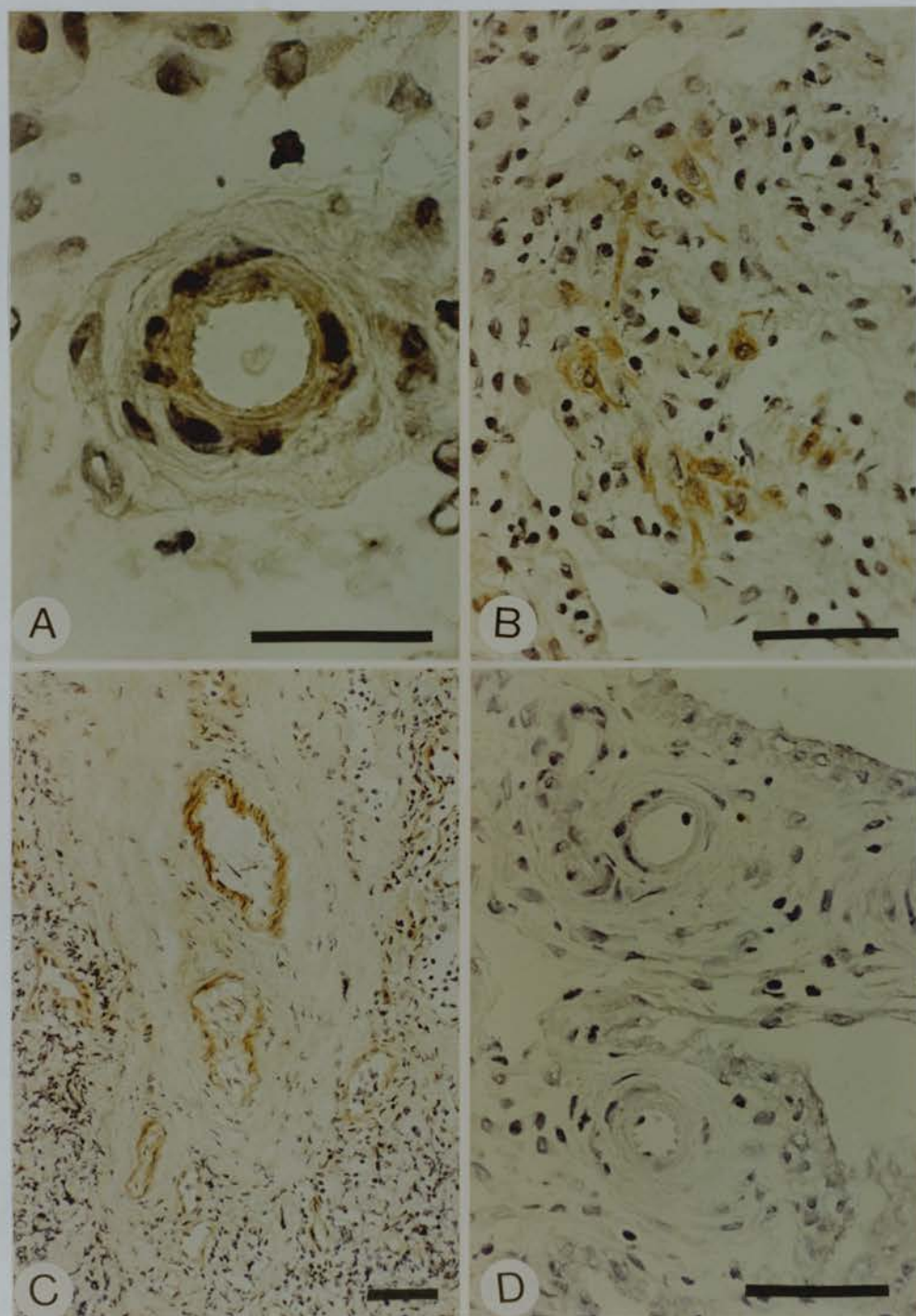


**Figure 16** MCP-1 immunoreactivity in human endometrium. As for IL-8, immunostaining for MCP-1 was primarily localised to the perivascular cells in non-pregnant and pregnant endometrium. (A) MCP-1 immunoreactivity was prominent in endometrium collected during the proliferative phase. (B) A marked reduction in staining intensity was detected in the ovulatory phase, whilst (C) premenstrual phase endometrium and (D) first trimester decidua exhibited strong MCP-1 immunostaining in the perivascular cells of all vessels. Scale bars = 50µm, v = vessel.



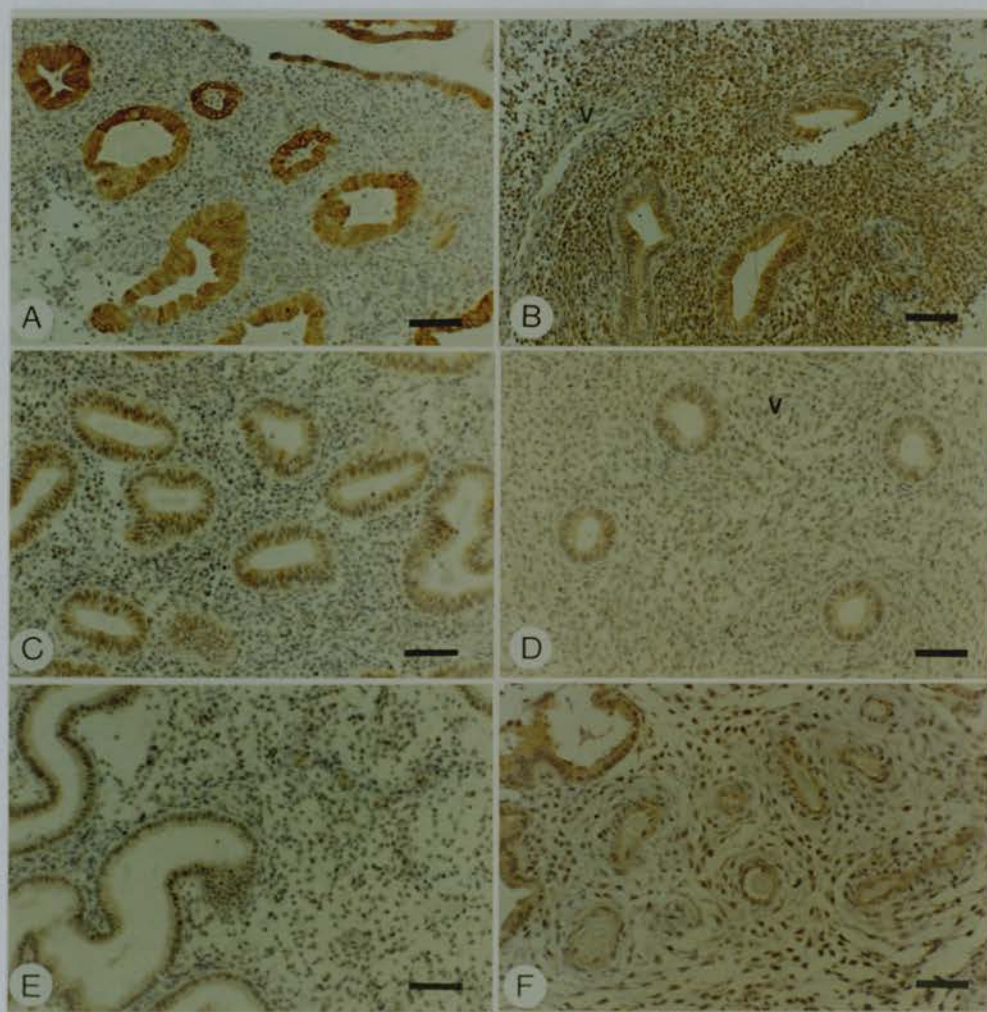


**Figure 17** MCP-1 immunoreactivity in human endometrium. (A) The specificity of the perivascular immunostaining is demonstrated in this photomicrograph taken at high magnification. (B) A distinct population of stromal cells were found to exhibit immunostaining for MCP-1 in some decidual samples. (C) Tonsil sections were included as a positive control, with immunostaining generally limited to blood vessels. (D) A negative control was included, by the pre-absorption of primary antibody with synthetic MCP-1 peptide, resulting in an absence of staining. For figure (A), scale bar = 25 $\mu$ m, all others scale bar = 50 $\mu$ m.



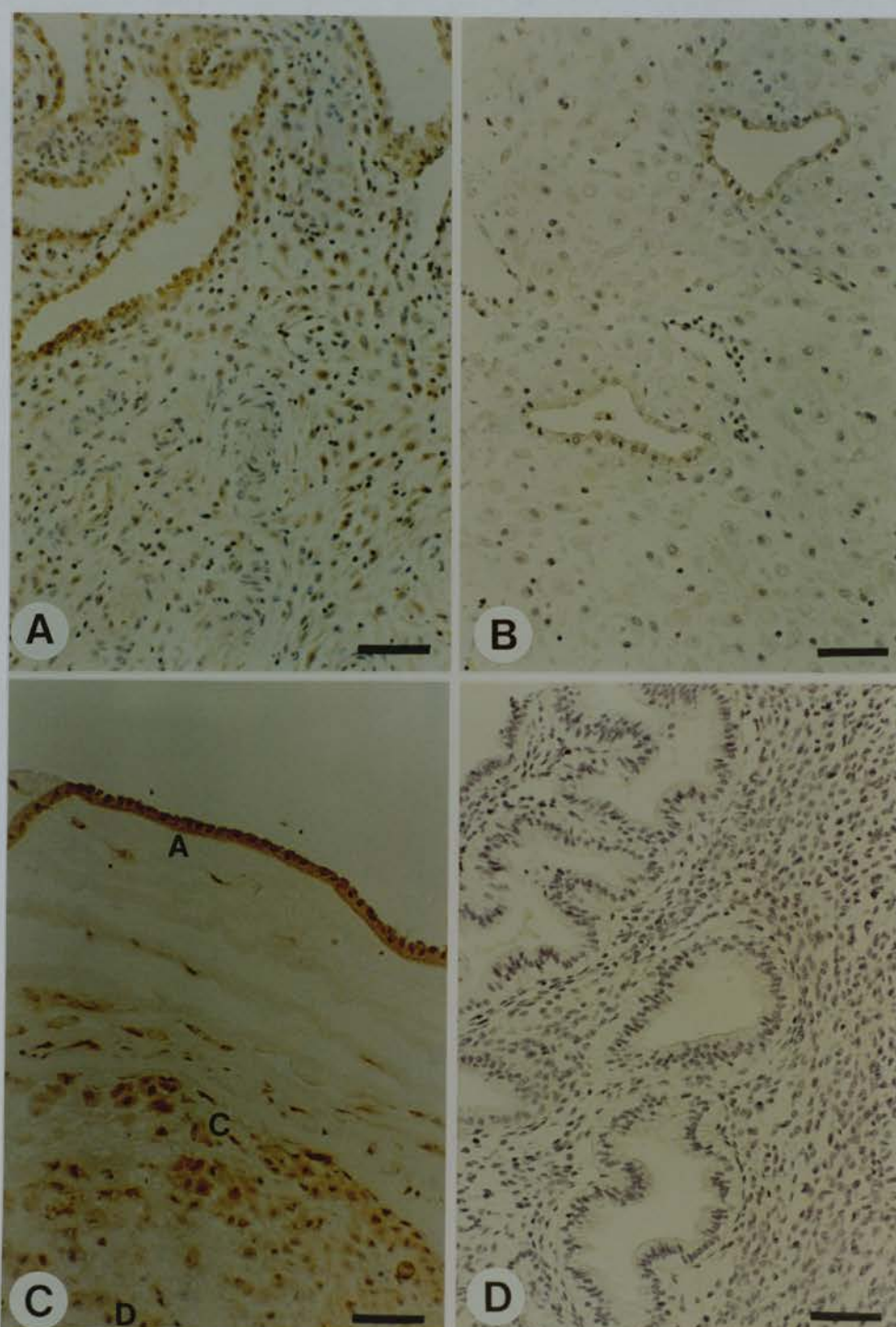
**Figure 18** COX-2 immunoreactivity in human endometrium. Immunoreactivity was generally localised to the glandular epithelium and blood vessels of non-pregnant and pregnant endometrium. Maximal COX-2 immunostaining was detected in menstrual phase endometrium, either (A) restricted to glandular epithelium or (B) present in glandular epithelium, stromal cells and the vasculature. (C) Glandular immunoreactivity was detected in proliferative endometrium, (D) which was markedly reduced in the periovulatory phase. (E) Faint perivascular immunostaining accompanied glandular immunoreactivity during the mid secretory phase. (F) Premenstrual endometrium exhibited intense immunostaining for COX-2 in both epithelial glands and perivascular cells.





**Figure 19** COX-2 immunoreactivity in human endometrium. COX-2 staining intensity and distribution was variable in decidua collected during the first trimester of pregnancy. It was interesting that regions exhibiting strong immunoreactivity generally displayed characteristics of secretory phase endometrium (**A**), whilst fully decidualised endometrium (**B**) possessed lower levels of immunoreactivity, limited to the glandular epithelium. (**C**) Term fetal membranes collected from women undergoing active labour were included as a positive control and exhibited COX-2 immunostaining in amniotic epithelium A, chorionic trophoblast, C and maternal decidua, D. (**D**) Secretory phase endometrium treated with rabbit immunoglobulins at a matching concentration to the primary antibody served as the negative control and possessed no immunostaining. Scale bars = 50µm.





## **IL-8 Immunohistochemistry**

The intensity of IL-8 immunostaining varied throughout the menstrual cycle (figure 20). Low levels were detected in the perivascular cells in the menstrual and proliferative phases of the menstrual cycle. A distinct but non-significant reduction in staining intensity was observed in the periovulatory phase and levels remained low in the early and mid secretory stages. Immunostaining levels increased slightly in the early and mid secretory phase, but a highly significant increase ( $p < 0.001$ ) in staining intensity was apparent in the late secretory phase in comparison with the ovulatory and early secretory phase. In the decidua, low levels of IL-8 immunoreactivity were observed. The average immunoreactivity was significantly ( $p < 0.01$ ) lower than that observed premenstrually and was consistent with the expression levels of IL-8 in the mid secretory phase.

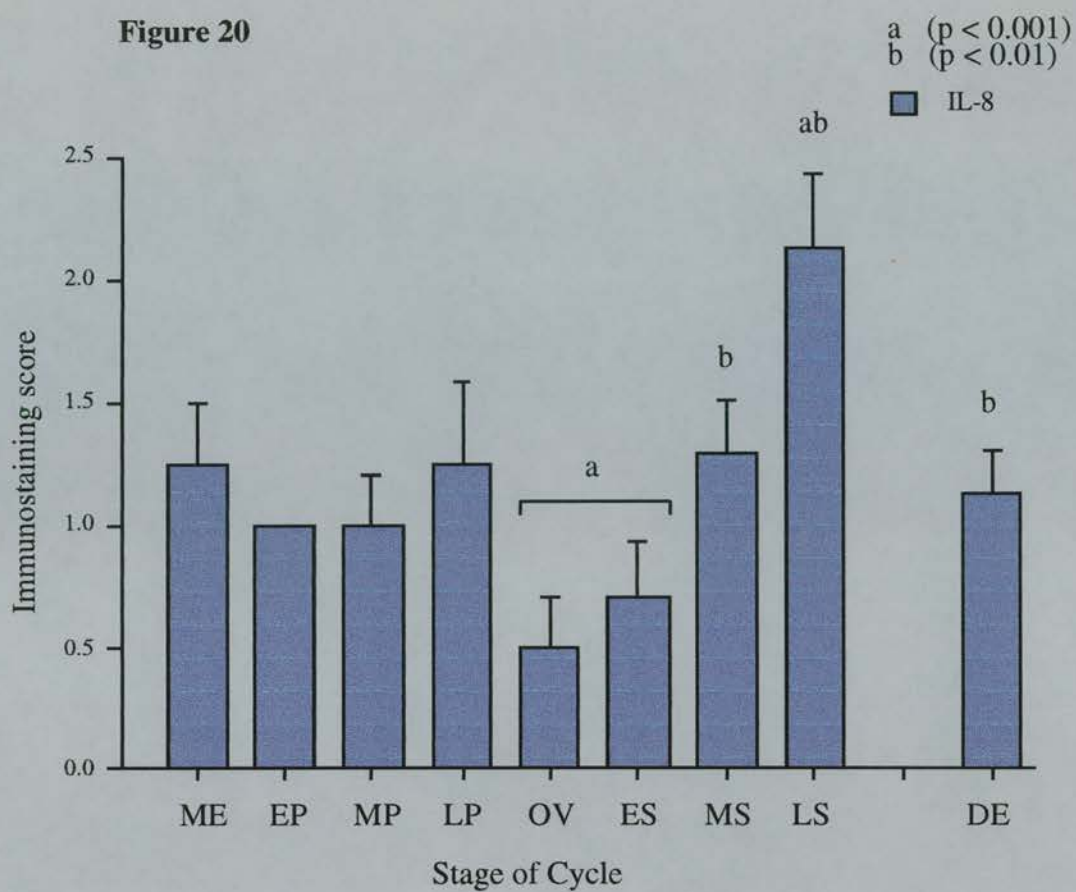
## **MCP-1 Immunohistochemistry**

MCP-1 immunoreactivity was detected in a cyclical pattern during the menstrual cycle (figure 21). Throughout the proliferative phase MCP-1 immunostaining was relatively intense. As for IL-8, there was a clear reduction in staining intensity in the periovulatory phase. In the premenstrual or late secretory phase, however, immunostaining levels increased significantly ( $p < 0.05$ ) compared to the periovulatory stage. Strong perivascular immunoreactivity for MCP-1 persisted in first trimester decidua.

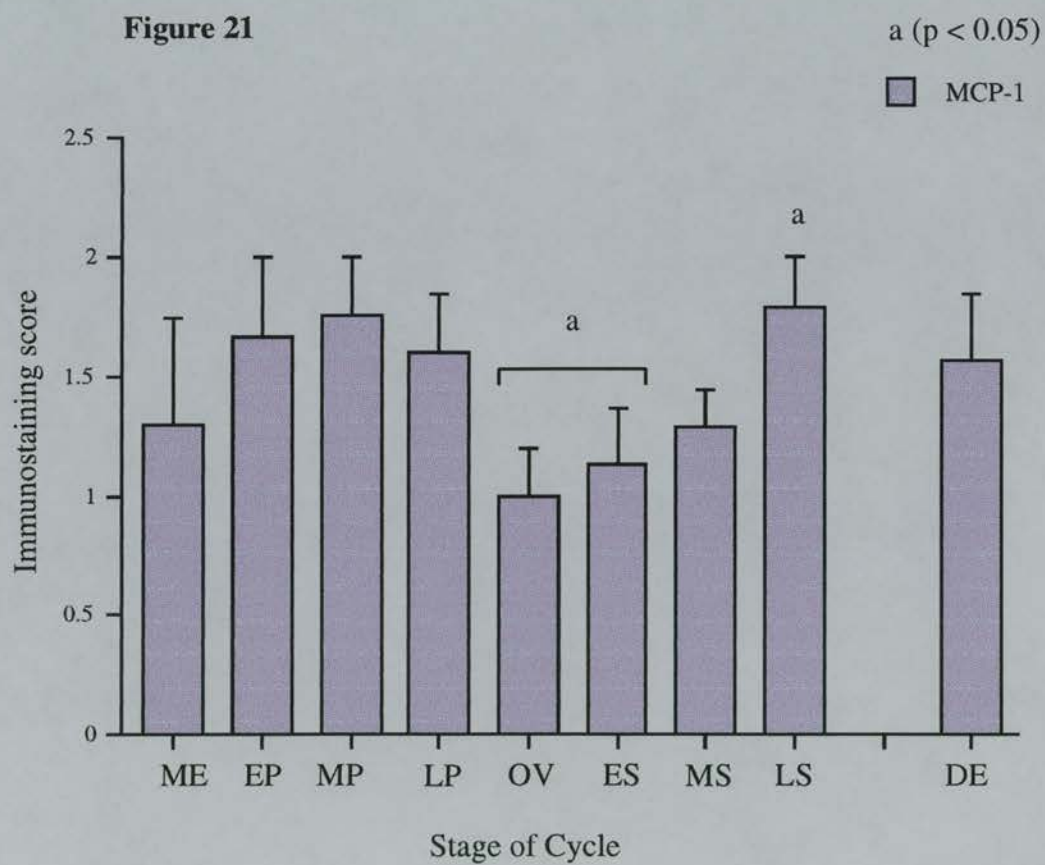
**Figure 20** IL-8 immunoreactivity throughout the menstrual cycle and in early pregnancy. A highly significant increase in immunostaining occurs in the premenstrual phase compared to levels in the ovulatory and early secretory phases ( $p < 0.001$ ) and the mid secretory phase ( $p < 0.01$ ). In early pregnancy, significantly lower levels of immunoreactivity were detected than premenstrually ( $p < 0.01$ ). Significant differences between groups are denoted by matching letters. Y axis error bars represent the standard error of the mean (SEM).

**Figure 21** MCP-1 immunoreactivity throughout the menstrual cycle and in early pregnancy. A significant increase in immunoreactivity levels are apparent in the premenstrual phase with respect to the ovulatory and early secretory phases ( $p < 0.05$ ). Significant differences between groups are denoted by matching letters. Y axis error bars represent the standard error of the mean (SEM).

**Figure 20**



**Figure 21**



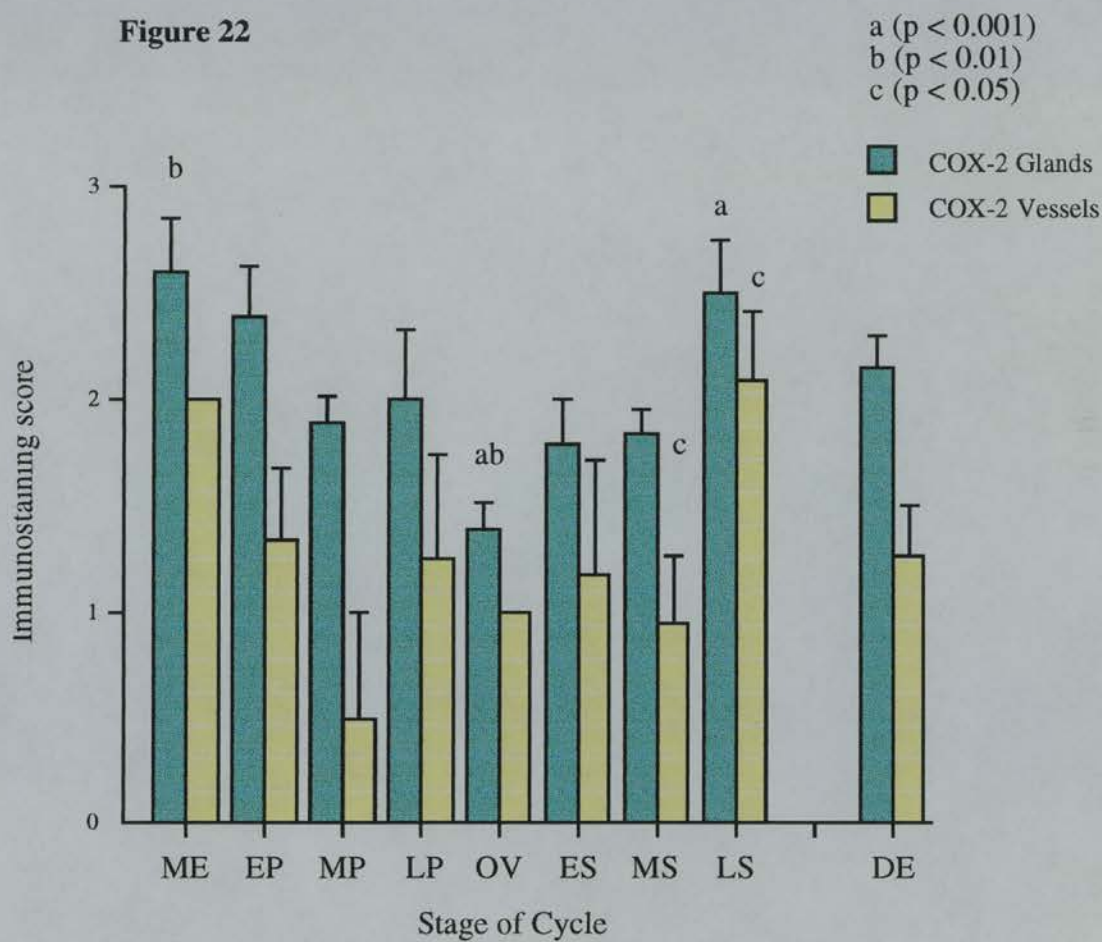
## **COX-2 Immunohistochemistry**

COX-2 immunoreactivity was observed at all stages of the menstrual cycle (Figure 3). Glandular epithelium and blood vessels exhibited intense heterogeneous immunostaining in the menstrual phase. In the proliferative stage, levels of immunoreactivity decreased slightly and lower levels still were detected in the ovulatory phase. This pattern continued through the early and mid secretory phases and a significant increase in COX-2 immunoreactivity was observed in the late secretory phase (Glandular  $p < 0.01$ , vessels  $p < 0.05$ ). The degree of glandular immunoreactivity was for COX-2 was variable in early decidua, with less immunostaining detected in regions displaying characteristics of fully decidualised endometrium (compared to secretory type endometrium), whilst perivascular staining appeared to be reduced (not significant).

**Figure 22** COX-2 immunoreactivity throughout the menstrual cycle and in early pregnancy. Glandular immunostaining is represented by filled bars and perivascular immunostaining by white bars. Glandular immunoreactivity was significantly elevated in the menstrual ( $p < 0.01$ ) and premenstrual phases ( $p < 0.001$ ) compared to the ovulatory phase. Perivascular immunostaining followed a similar pattern, with a significant increase in the premenstrual phase ( $p < 0.05$ ) with respect to the early secretory phase. Significant differences between groups are denoted by matching letters. Y axis error bars represent the standard error of the mean (SEM).



**Figure 22**





## Semi-quantitative RT-PCR

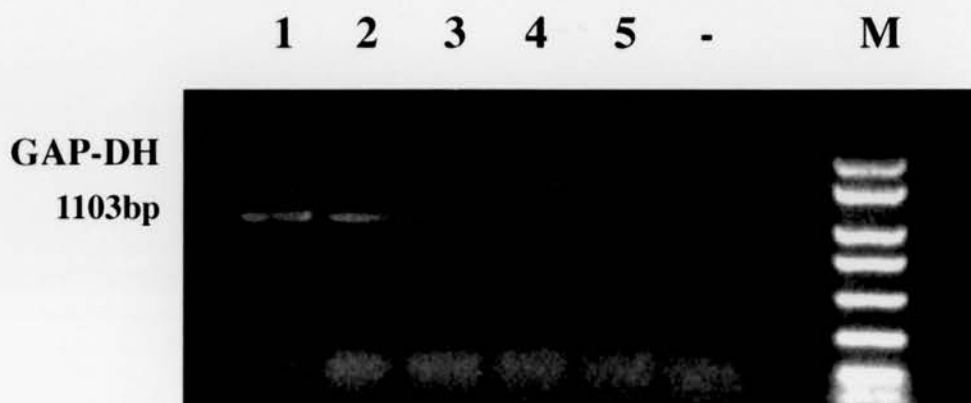
All RNA samples selected for GAP-DH RT-PCR were found to contain viable RNA. The standard curve generated the equation  $y = 0.005x - 0.003$ , where  $x$  is the amount of RNA template loaded and  $y$  is the absorbance of the probe-product complex at 450nm (figure 23). RNA concentrations were calculated and the RT-PCR for GAP-DH was repeated with 60ng. Bands of equal intensity corresponding to the amplified portion of GAP-DH cDNA were detected by gel electrophoresis, indicating equal product yield and RNA loading and this was confirmed by ELISA (all samples 60 +/- 10ng).

RT-PCR for IL-8, MCP-1 and COX-2 resulted in positive signal of expected molecular weight (IL-8 - 298bp, MCP-1 - 210bp, COX-2 - 350bp)(figure 24). The identity of the IL-8 and MCP-1 amplicons was confirmed by subcloning into expression vector pGEM®-T Easy Vector (Promega Ltd., Southampton, U.K.) and sequencing using the dideoxy chain termination method. Product yield was assessed by ELISA and expressed as a ratio of sample signal : Quality Control (QC) signal.

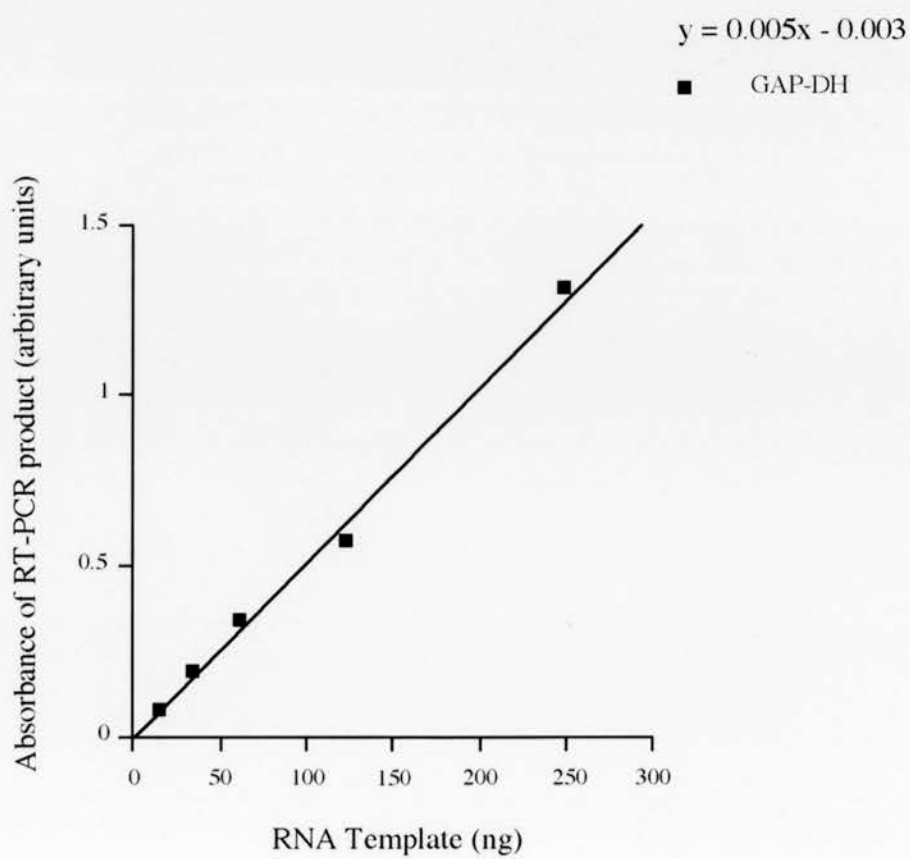
High levels of expression for IL-8 and COX-2 were observed in the menstrual phase samples. In contrast, MCP-1 mRNA levels were low in the majority of menstrual samples examined. Throughout the proliferative and early-mid secretory phases, relatively low numbers of mRNA transcripts were detected (statistically significant with respect to menstrual phase for IL-8 ( $p < 0.0001$ ) and COX-2 ( $p < 0.02$ )), although a trend of a slight increase was apparent in the late secretory endometrium (not significant). Low levels of COX-2 mRNA were generally detected in first trimester decidua (not significant), whereas the IL-8 and MCP-1 signal was slightly elevated when compared to the proliferative, ovulatory / early secretory and mid secretory signals (IL-8  $p < 0.05$ ; MCP-1  $p < 0.05$ ). A large degree of variability was observed at many stages of the cycle for COX-2 mRNA levels, which is reflected by the error bars on the histogram representing the standard error of the mean (SEM).

**Figure 23** Standard curve for GAP-DH RT-PCR. (A) Standards amplified for GAP-DH (1 = 250ng - 5 = 17.5ng) analysed by gel electrophoresis. (B) Product yield was assessed by ELISA and amount of RNA template (x) was plotted against the absorbance of the RT-PCR product (y). This generated the equation  $y = 0.005x - 0.003$ . A linear relationship between amount of starting template (RNA) and amount of product generated was obtained, and the loading of subsequent RT-PCR was adjusted to ensure that all reaction tubes contain 60ng of RNA template.

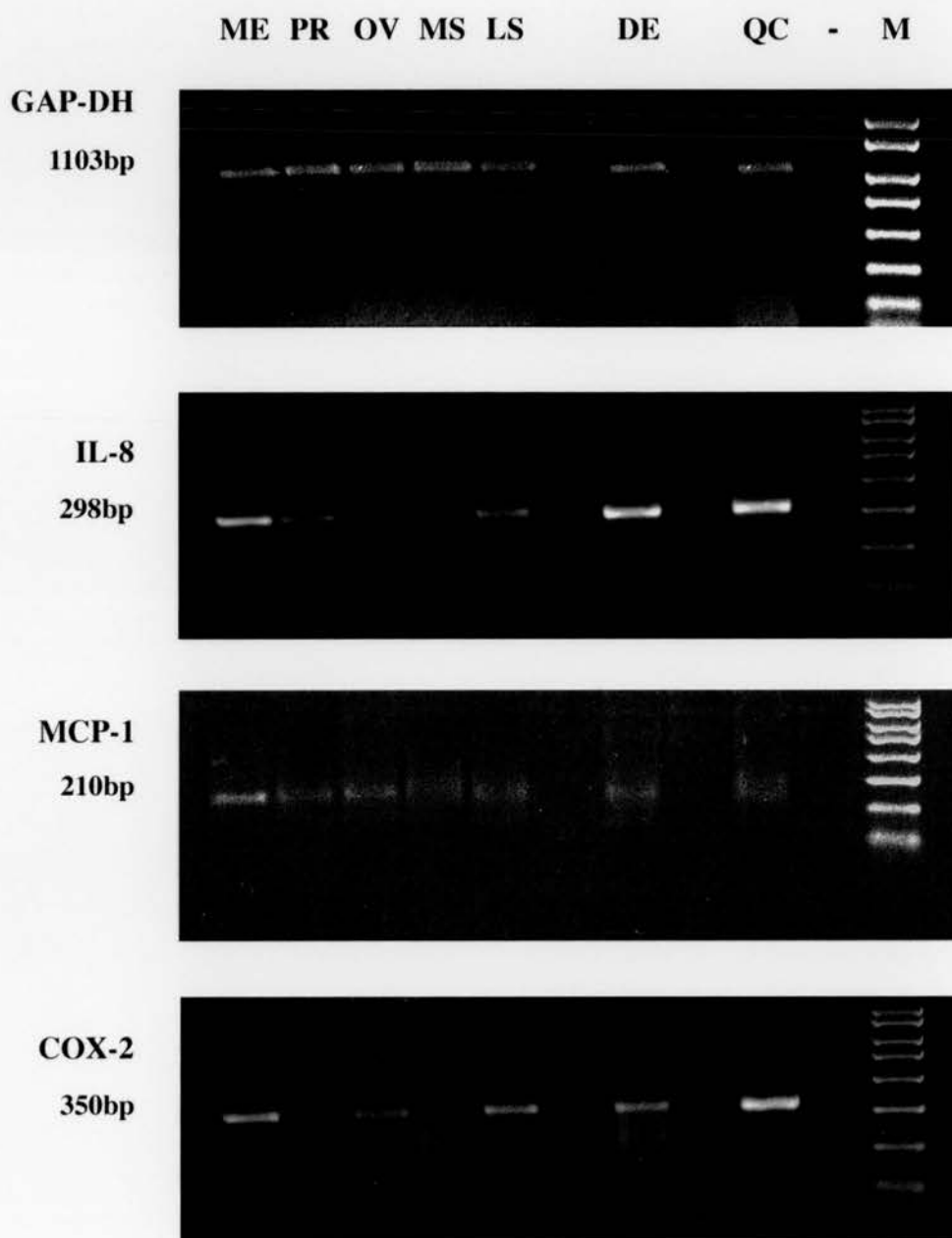
**A**



**B**



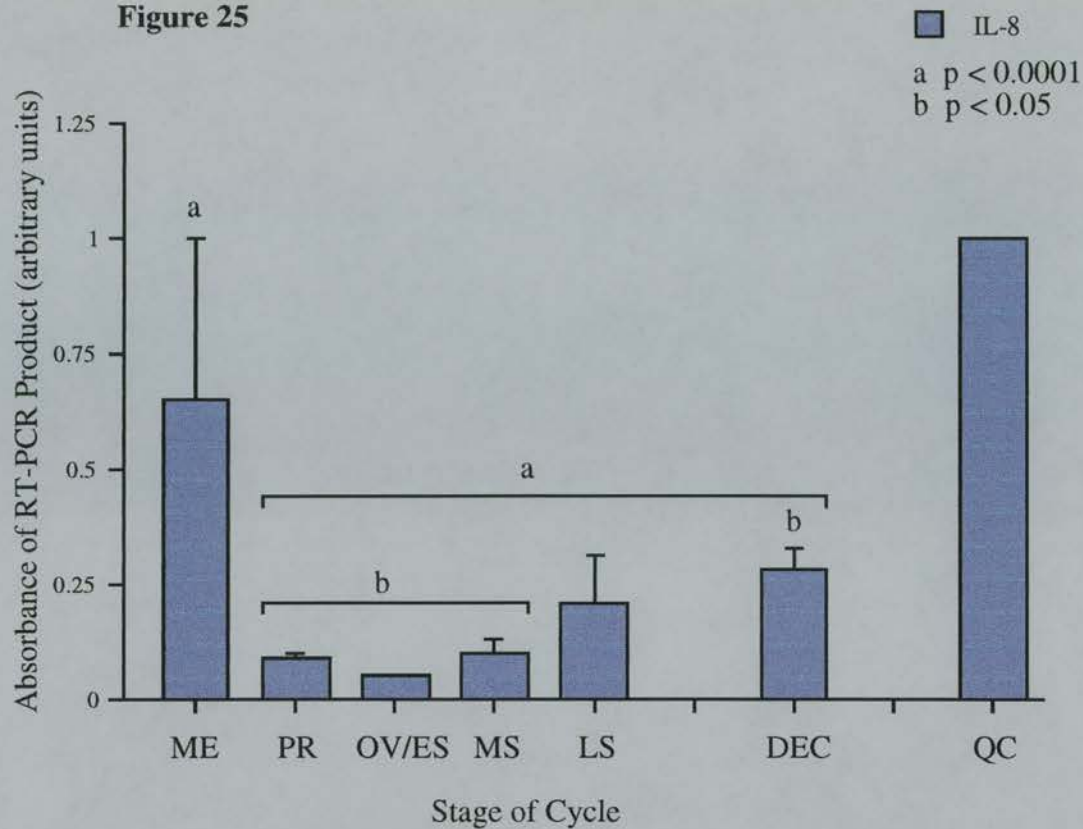
**Figure 24** Expression of IL-8, MCP-1 and COX-2 mRNA in human endometrium. RT-PCR products from representative RNA samples throughout the menstrual cycle are shown (ME = menstrual, P = proliferative, OV/ES = ovulatory / early secretory, MS = late secretory, LS = late secretory, DE = decidua). A quality control (QC) of menstrual phase endometrium was included in each run to allow consistency between individual RT-PCRs and to standardise subsequent ELISAs. The upper panel demonstrates samples (60ng) amplified for GAP-DH, indicating the equal loading of RNA into RT-PCRs for the inflammatory mediators. A signal of 298bp was detected, corresponding to the portion of the IL-8 gene targeted, with strongest expression in the menstrual and late secretory phases and first trimester decidua samples. For MCP-1, a signal of 210bp was detected by gel electrophoresis, which was strongest in the menstrual phase, but present at all stages of the menstrual cycle and early pregnancy. A single band of 350bp was detected representing the COX-2 amplicon, with strongest expression in the menstrual and late secretory phases and first trimester decidua.



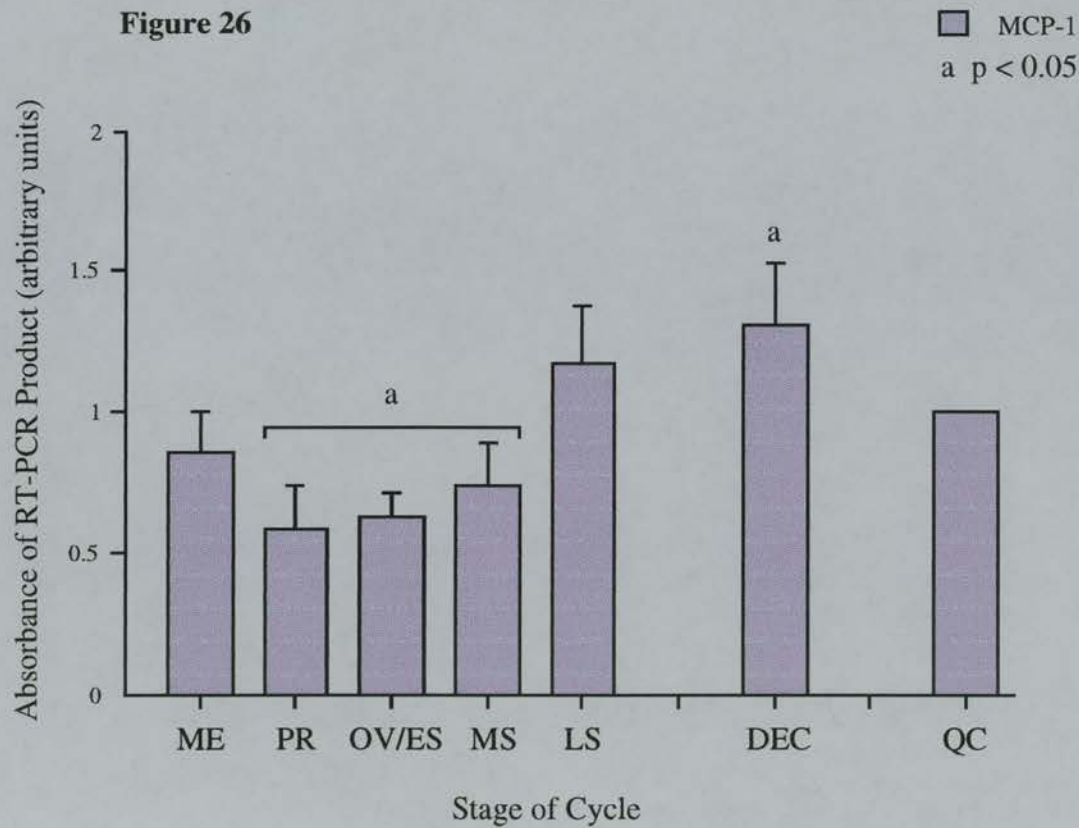
**Figure 25** Expression of IL-8 mRNA in human endometrium. Highly elevated levels of IL-8 mRNA transcripts were present in menstrual phase endometrium (d1-4), which was highly significant ( $p < 0.0001$ ) when compared to all other stages. With cessation of menses, levels of mRNA fell and were barely detectable in the proliferative, periovulatory / early secretory, mid and late secretory phases. The signal increased slightly in the late secretory endometrial samples and similar levels were detected from first trimester decidual samples (significant elevation from proliferative, periovulatory / early secretory and mid secretory phases  $p < 0.05$ ).

**Figure 26** Expression of MCP-1 mRNA in human endometrium. Slight variations in MCP-1 mRNA levels were detected from samples throughout the menstrual cycle. The number of MCP-1 transcripts in the late secretory phase and during menses appeared to be increased with respect to the proliferative, periovulatory / early secretory and mid secretory phases (not significant). Elevated MCP-1 levels were detectable in first trimester decidua ( $p < 0.05$ ) when compared to proliferative, periovulatory / early secretory and mid secretory phases.

**Figure 25**



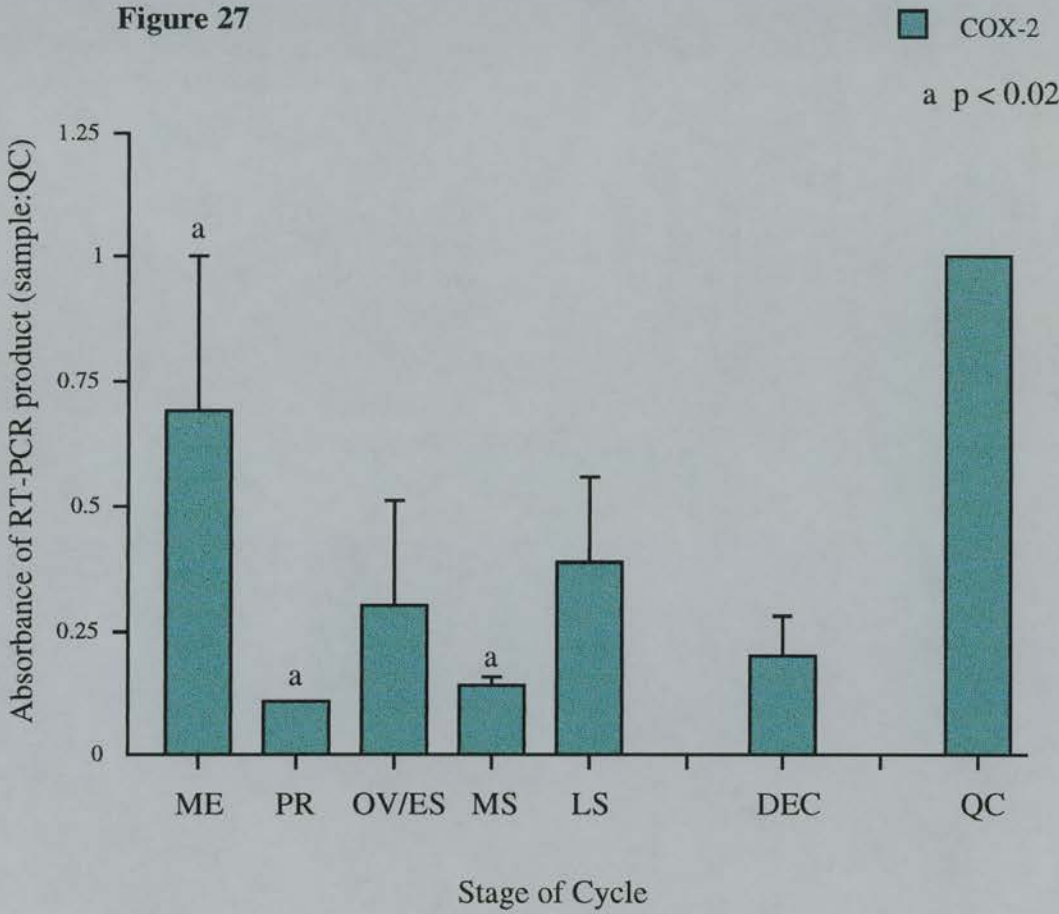
**Figure 26**





**Figure 27** Expression of COX-2 mRNA in human endometrium. Strong expression of COX-2 was detected by RT-PCR in the menstrual phase endometrium. Levels of COX-2 mRNA transcripts were decreased significantly ( $p < 0.02$ ) in the proliferative and mid secretory phases. Slightly elevated levels were detectable in the periovulatory / early secretory and late secretory phases. Low numbers of COX-2 mRNA transcripts were present in first trimester decidua.

Figure 27



### 3.4 Discussion

MCP-1, IL-8 and COX-2 were localised in the endometrium and decidua by immunohistochemistry. The localisation of these chemotactic agents to the perivascular cells in both pregnant and non pregnant endometrium is consistent with a putative role in the recruitment of specific leukocytes from the vasculature. Elevated levels of chemokine immunoreactivity were detected in the premenstrual phase of the menstrual cycle. Supporting evidence was obtained from RT-PCR studies, with particularly strong expression of IL-8 and COX-2 mRNA in the menstrual phase. These findings are supportive of the hypothesis that migration of leukocytes from the peripheral circulation contributes to the significant leukocyte accumulation observed premenstrually. Furthermore, the co-localisation of COX-2 in the perivascular cells reinforces the proposed interaction of prostaglandins and chemokines in the local inflammatory response.

The exact cell types in the perivascular region which exhibit immunoreactivity have not been characterised. Endothelial cells, fibroblasts and smooth muscle cells are present in this location, and all have been demonstrated to be capable of chemokine production (see reviews (Matsushima et al., 1989; Baggiolini et al., 1994)). Furthermore, specialised myofibroblasts may be responsible for chemokine and COX-2 expression (McGrath, 1982; Valentich et al., 1994). Future studies could address this question utilising double immunohistochemistry techniques for chemokines and  $\alpha$ -smooth muscle actin, desmin or an endothelial cell marker (CD34 / CD31) (Gabbiani, 1992; Abberton et al., 1996). It is possible that the detection of the chemokines in this position is due to their immobilisation on the endothelial cells, via proteoglycans or the Duffy antigen, which is necessary for leukocyte recruitment (Witt et al., 1994; Middleton et al., 1997). Additional immunoreactivity was identified in a subpopulation of stromal cells in some decidual sections. These observations may represent a population of leukocytes with the potential for chemokine production.

Examination of tissues obtained throughout the menstrual cycle and in early pregnancy revealed a cyclical pattern in the intensity of immunoreactivity for all three mediators. Most noticeable was the distinct reduction in staining intensity around the time of ovulation, extending into the early secretory phase. This coincides with the initiation of progesterone production from the corpus luteum and peak oestrogen production. At this stage the endometrium is most responsive to the effects

of oestrogen and progesterone due to maximal steroid receptor expression (Snijders et al., 1992). Furthermore the perivascular cells identified as the site of chemokine production strongly express progesterone receptors throughout the cycle (Perrot-Applanat et al., 1988; Bouchard et al., 1991). Experimental evidence has demonstrated that MCP-1 secretion by monocytes and fibroblasts in culture is inhibited by oestrogen (Kovacs et al., 1993; Kovacs et al., 1996). A similar regulation may occur *in vivo*, although the levels of MCP-1 immunoreactivity detected during the proliferative phase fail to support this. The levels of chemokine and COX-2 immunoreactivity detected remain low through the early and mid secretory phases, until the late secretory phase when a significant elevation in expression is apparent. At this stage in the non-pregnant cycle, regression of the corpus luteum results in a drop in progesterone levels. If pregnancy occurs, progesterone levels are maintained and this generally corresponds to a lower level of immunoreactivity for the mediators.

It therefore appears that the staining intensity for all three mediators is related to the prevailing steroid hormone environment, with a reduction in the presence of progesterone and an increase when progesterone concentrations decline premenstrually. These observations are consistent with a downregulation of the chemokines and COX-2 by progesterone, such as has been demonstrated *in vitro* (Ito et al., 1994; Kelly et al., 1994; Ishihara et al., 1995). This immunohistochemical evidence of a co-localisation of chemokine and COX-2 producing cells with progesterone receptors suggests a receptor mediated indirect effect of progesterone in regulating leukocyte migration. This would represent an anti-inflammatory action of progesterone, in the suppression of a local inflammatory response at the time when fertilisation and implantation may occur. This is supported by the recent demonstration of inflammation and hyperplasia in the uterus of a homozygous mouse deficient for progesterone receptors (Lydon et al., 1995).

The phenotypically unique natural killer-like LGLs (CD56<sup>+</sup>CD16<sup>-</sup>) are the predominant leukocyte subtype in the late secretory endometrium and first trimester decidua. Their origin is uncertain, but it has been suggested that a precursor form may be recruited from peripheral blood into the endometrial stroma, where a subsequent activation / differentiation occurs (King et al., 1991). A putative precursor cell type are the CD56<sup>+</sup>CD16<sup>-</sup> agranular cells which comprise around 1% of the circulating NK cells (Lanier et al., 1986). Chemotactic activity for NK cells has been attributed to MCP-1 (Allavena et al., 1994). Further investigation is necessary to determine whether MCP-1 may play a role in the accumulation of LGLs

in the mid-late secretory phase and in the first half of pregnancy. IL-8 has also been implicated in the chemotaxis of lymphocytes (Larsen et al., 1989a), and this has been linked to the LGL infiltrate in the endometrium (Casey et al., 1993). However, it has recently been proposed that only the C-C chemokines (including MCP-1) and not the C-X-C subfamily, which include IL-8, possess chemotactic ability for T lymphocytes (Roth et al., 1995).

The co-localisation of IL-8 and COX-2 in the endometrium reinforces the synergistic action of PGE in the IL-8 stimulated recruitment of neutrophils (Foster et al., 1989; Rampart et al., 1989; Colditz, 1990). Furthermore, as MCP-1 and IL-8 are closely related chemokines, it is possible that PGE may also have an enhancing effect on monocyte infiltration, since the synergistic effect described in all publications involves a vasoactive property of PGE. Prostaglandin activity is also regulated by the locally produced metabolising enzyme prostaglandin dehydrogenase (PGDH). A positive regulation by progesterone has been demonstrated by the reduced immunoreactivity for PGDH following antigestogen administration in early pregnancy (Cheng et al., 1993b). Also observed was increased immunoreactivity for PGE<sub>2</sub> around the small blood vessels in the endometrium (Cheng et al., 1993a). Thus progesterone may affect local prostaglandin concentrations by modulating the enzymes responsible for both their production and metabolism.

The localisation of COX-2 primarily to the glandular epithelium is supportive of the *in vitro* evidence that glands are the major site of prostaglandin synthesis in the endometrium (Rees et al., 1982; Lumsden et al., 1984; Smith et al., 1988). Furthermore, whilst the endometrium retains the ability to synthesise prostaglandins in response to stimuli throughout the menstrual cycle, higher levels of prostaglandins are released from isolated glands in the proliferative than in the secretory phase (Smith et al., 1988). First trimester decidua appears to have a reduced capacity for arachidonic acid metabolism, thus basal levels of PGs are low (Maathuis et al., 1978). This is consistent with a stimulation of prostaglandin production by oestrogen (Abel et al., 1980), potentially through cyclooxygenase upregulation (Jun et al., 1996), and a downregulation by progesterone. The variable nature of COX-2 immunostaining in first trimester decidua may be explained by the different histological appearance of tissues collected at this stage. The process of decidualisation is not complete by 8-10 weeks of pregnancy and this is reflected by the collection of tissue displaying clear characteristics of decidua, and other regions, presumably collected from deeper regions of decidua (decidua spongiosa), which resemble secretory endometrium (Bell, 1991). Immunoexpression of COX-2 was

much higher in the secretory type endometrium, whilst minimal immunoreactivity was generally detected in true decidua.

The present study has therefore identified and immunolocalised the inflammatory mediators MCP-1, IL-8 and COX-2 to the perivascular cells in endometrium and decidua. A cyclical pattern of immunoreactivity has been observed, with maximum expression of MCP-1, IL-8 and COX-2 occurring perimenstrually and coinciding with the large increase in leukocytes in the endometrial stroma at this time. These observations support a role for steroid-regulated chemokines in the mechanisms determining leukocyte accumulation in endometrium and decidua.

## **CHAPTER 4**

### ***IN VIVO* PERTURBATION OF LOCAL PROGESTOGEN ENVIRONMENT: EFFECT ON ENDOMETRIAL CHEMOKINE AND COX-2 EXPRESSION**



## 4.1 Introduction

The expression and localisation of locally active factors IL-8, MCP-1 and COX-2 in the endometrium has been demonstrated in the previous chapter. Both chemokines were immunolocalised to the perivascular cells of endometrial and decidual vessels. Similarly, COX-2 protein was detected in the vasculature, and further immunoreactivity in glandular epithelium was described. The temporal expression pattern of all three inflammatory mediators indicates a role in leukocyte recruitment to the endometrium premenstrually and thus potentially in the initiation of menstruation. The interesting cyclical pattern observed for both protein and mRNA levels has prompted further investigation into the role of the sex steroid hormone, progesterone, in the regulation of these paracrine factors.

A further aspect of these studies has been to consider the clinical application of these observations in the context of progesterone only contraception and the impact of progesterone on endometrial function. Modification of progesterone levels is a useful tool in gynaecology and obstetrics, particularly in the development of contraceptives and for the termination of pregnancy (Baird, 1993). High continuous production of progesterone is essential for the establishment and maintenance of pregnancy, and inappropriately elevated concentrations or withdrawal can prevent or terminate a pregnancy (Csapo et al., 1973). Women under 9 weeks gestation may terminate their pregnancy by taking the antigestogen mifepristone (RU486) followed by a prostaglandin analogue 24 hours later (Van Look et al., 1989). RU486 effectively blocks the action of progesterone by occupying receptor sites, yet not effecting a cellular response. Recent and ongoing work has been researching into the use of RU486 as a contraceptive (Baird, 1993), particularly focusing on the development of a once a month or lose dose daily pill (Gemzell-Danielsson et al., 1994; Cameron et al., 1995; Cameron et al., 1996; Cameron et al., 1997; Gemzell-Danielsson et al., 1997a; Gemzell-Danielsson et al., 1997b).

Progesterone administration, either orally or as a slow release mechanism are presently used for contraceptive purposes. The oral contraceptive, combining the effects of oestradiol and progesterone, functions in a number of ways to prevent pregnancy, through the inhibition of ovulation, alteration of cervical mucus, disruption of the endometrial receptivity in the 'implantation window' (Kubba et al., 1993; Somkuti et al., 1996). Recently, slow release subdermal implants (Norplant®, Leiras, Finland) and an intrauterine system (Lng-IUS; Mirena, Leiras, Finland)

incorporating the perturbation of the steroid environment, have been licensed in this country. Both preparations release levonorgestrel (Lng), a progesterone analogue. Both are highly effective as contraceptives, and the Lng-IUS has the added advantage of dramatically reducing menstrual blood loss and thus proving useful as a treatment for menorrhagia and dysmenorrhoea (Nilsson et al., 1982; Andersson et al., 1990). Ovulation is not inhibited in most users of Norplant and Lng-IUS (Nilsson et al., 1984; Luukkainen et al., 1990), the contraceptive effect is instead mediated through the prevention of proliferation and differentiation of the endometrium and thickening of cervical mucus (Silverberg et al., 1986; Barbosa et al., 1990). The main reason for discontinuation of use of progestin only contraceptives, however, is the breakthrough bleeding commonly experienced by users (Sivin et al., 1983; Odell et al., 1990; Findlay, 1996). This is associated with decidual transformation of the endometrium and infiltration of leukocyte subpopulations (Sheppard, 1987). Important research is currently underway to understand this unacceptable side effect associated with continuous exposure of the endometrium to progestins (Critchley et al., 1998a; Wang et al., 1997; Critchley et al., 1998b).

Progesterone withdrawal triggers an inflammatory type response, including the accumulation of leukocytes, stromal oedema, elevated prostaglandin and cytokine production and increased production of active MMPs (Finn et al., 1984; Salamonsen et al., 1996b). All are believed to have an integral role in the initiation of menstruation (Kelly et al., 1994). The cellular mechanisms behind breakthrough bleeding associated with progestogen only contraception and termination of pregnancy by administration of antigestogens and prostaglandins are unknown. Antigestogen administration during the luteal phase of the normal menstrual cycle or in early pregnancy induces bleeding (Herrmann et al., 1985; Swahn et al., 1988; Swahn et al., 1990), and retards endometrial development (Gemzell-Danielsson et al., 1997). First trimester decidua collected after withdrawal of progesterone shows reduced immunoreactivity for PGDH (Cheng et al., 1993b), resulting in elevated concentrations of PGE<sub>2</sub> around blood vessels (Cheng et al., 1993a). Sensitivity of the decidua to exogenous PGE is also increased (Brooks et al., 1990). RU486 treatment *in vitro* stimulates endometrial and decidual cell PG synthesis (Kelly et al., 1986; Smith et al., 1987), although the mechanism is unknown. Given the proposed role of chemokines and prostaglandins in normal menstruation, the question of their involvement in breakthrough bleeding and induced abortion is raised.

The present study investigates the effect that modification of progesterone concentrations has on local endometrial factors. The expression of IL-8, MCP-1 and COX-2 was examined by means of immunohistochemistry and RT-PCR in endometrium where the progesterone environment has been artificially perturbed. These studies include:

1. Model of progesterone withdrawal and maintenance *in vivo*, to simulate the onset of menses and provide an opportunity to study the earliest stages of pregnancy.
2. The pharmacological withdrawal of progesterone by RU486 administration at varying time points prior to surgical termination of first trimester pregnancy.
3. Endometrium constantly exposed to high local concentrations of progestin via an levonorgestrel releasing intra-uterine system (Lng-IUS).

## 4.2 Materials and Methods

Healthy fertile women aged between 20 and 48 years of age, with normal menstrual cycles (cycle length 25 - 35 days) were recruited to take part in these studies. All subjects were fertile and had not used any form of hormonal treatment in the previous three months. Ethical approval for each clinical study was obtained from Lothian Research Ethics Committee (reference: 1702/94/6/1) and each patient was required to give written informed consent. These studies were supported by project grants from the Medical Research Council (G94 06438PA and G96 20138) and The Wellcome Trust (044744/Z/95/Z).

### Study Groups:

Study 1 Endometrium from different stages of the menstrual cycle was collected from five groups of women:

- (i) Normal mid-secretory phase endometrium (day LH + 8 - 10), corresponding to peak serum progesterone concentrations (Control Group A, n = 5).
- (ii) Premenstrual phase endometrium. In order to time the samples accurately, 200mg of the progesterone analogue Cycloogest was administered from day LH + 8 for four days by vaginal pessary (Cyclogest [Hoechst] 200mg b.d.). Endometrial biopsies were collected 24 hours after stopping progesterone (Group B, n = 5).

Endometrium was also collected from a further cohort of women, 48 hours after exogenous progesterone withdrawal (Group C, n = 5).

- (iv) Pseudopregnant endometrium. The lifespan of the corpus luteum (CL) was extended by daily injections of hCG from day LH + 8 for 14 days, maintaining progesterone production, to simulate early pregnancy. Incremental doses of hCG ranging from 125 to 20,000 IU (Illingworth et al., 1990) were administered over the 14 days (Group D, n = 5).
- (v) First trimester decidua from women with tubal pregnancy, ectopic to the uterus. Women who had experienced vaginal bleeding were excluded from this study (Group E, n = 5).

**Table 6**      Table illustrating the different subgroups used in study 1.

STUDY 1 GROUPS	STAGE OF CYCLE	METHOD
A	Mid-secretory endometrium	Control
B	Premenstrual endometrium	24 hours after exogenous progesterone withdrawal
C	Premenstrual endometrium	48 hours after exogenous progesterone withdrawal
D	Pseudopregnant endometrium	"Rescue" of CL by hCG administration
E	Decidua without trophoblast invasion	Decidua from pregnancy ectopic to the uterus

**Study 2** Early pregnancy decidua was collected from women (35 - 56 days amenorrhoea) seeking termination of pregnancy, according to the 1967 Abortion Act. The effect of progesterone withdrawal on first trimester decidua was investigated by administration of the antigestogen mifepristone (RU486) prior to suction termination of pregnancy. Women were randomly allocated to six groups. An oral dose of 200mg of RU486 was given to groups II, II, IV and V (n = 7 in each) at 6, 12, 24 and 36 hours respectively before tissue collection. The control group I (n = 8) did not receive RU486. Decidua parietalis was biopsied as described earlier (section 2.21) and the absence of trophoblast invasion was confirmed by immunohistochemistry for cytokeratin (section 2.22).

**Study 3** This study involved the local exposure of the endometrium to high local concentrations of synthetic progestin via a levonorgestrel releasing intra-uterine system (Lng-IUS). Women (n = 14) were recruited for contraceptive purposes (n = 10) or for the treatment of menorrhagia (n = 4). The Lng-IUS (Mirena) consists of a plastic T-shaped frame, surrounded by a levonorgestrel containing cylinder. A polydimethylsiloxane membrane ensures a daily delivery of 20µg into the uterine cavity. The Lng-IUS was inserted in subjects in both the proliferative phase (n = 7) and the secretory phase (n = 7). An endometrial biopsy was taken prior to insertion of the Lng-IUS and further biopsies were collected at 1, 3, 6 and 12 months post insertion. This was therefore a longitudinal study design, with each patient acting as her own control.

### **Tissue Treatment**

For all study biopsies, the tissue was divided and a portion fixed overnight in 10% NBF to be processed into paraffin blocks, whilst another sample was frozen in OCT embedding medium, as for control endometrial biopsies. When enough tissue was collected for Study 1 patients (n = 15), a further portion was frozen in a cryotube and RNA was extracted as described previously (section 2.24). All biopsies were allocated a random code to enable immunostaining, scoring and statistical analysis to be conducted blindly and randomly.

### **Immunohistochemistry**

Immunostaining for IL-8, MCP-1 and COX-2 was conducted on all study tissues, using protocols described earlier (section 2.22). As far as was possible, samples from a particular study were stained in the same run, to exclude artefactual variation



in staining intensities. Analysis of staining was conducted blindly by two independent observers.

### **Semi- quantitative RT-PCR**

RNA was extracted from endometrium collected from 15 patients (n = 3 in each group) taking part in Study 1. Sample RNA was initially subjected to RT-PCR for GAP-DH to determine RNA concentration and enable equal loading of subsequent reactions (section 2.25). RT-PCRs for IL-8, MCP-1 and COX-2 were conducted and the product yield determined by ELISA (section 2.26).

## 4.3 Results

### Immunohistochemistry

Immunoreactive protein for IL-8 and COX-2 was detected in the endometrium from all study groups. As in the non-pregnant and pregnant endometrium (Chapter 3), all were localised to a perivascular position, with additional staining for COX-2 in detected in glandular epithelium.

Study 1 Histological features of the tissue sections were consistent with the stage of the cycle (LH dating). Biopsies collected during the secretory phase possessed characteristics of decidualisation, including LGL accumulation, and spiral arteriole formation. Premenstrually timed biopsies (Group C) exhibited signs of tissue breakdown. Decidua from groups D and E was described as late secretory, with a greater degree of decidualisation evident when the gestation was ectopic.

Positive immunoreactivity for IL-8 was detected in a perivascular position in all tissue samples examined (figure 28). Intense perivascular immunostaining was observed in premenstrual groups B and C, with additional staining evident in glandular and stromal compartments 48 hours post progesterone withdrawal, in tissues exhibiting features of tissue breakdown. COX-2 immunoreactivity was present in endometrium from all study groups (figure 29). Maximal immunostaining was detected in premenstrual endometrium (group C), with noticeable heterogeneous glandular immunostaining, as detected in menstrual phase biopsies (Chapter 3, section). COX-2 immunoreactivity was lowest in early decidua, pharmacologically maintained by hCG administration.

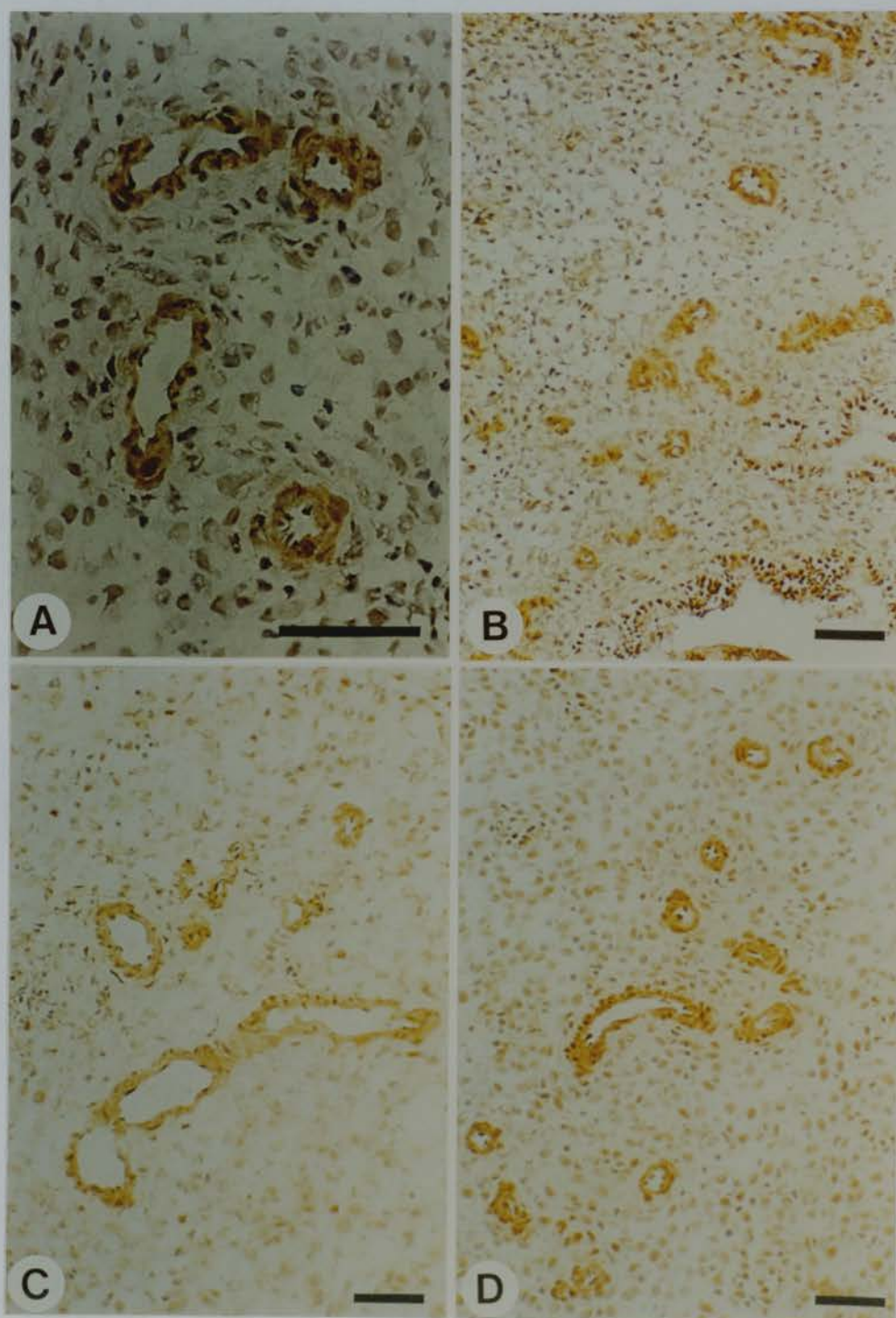
### Serum Progesterone and Oestradiol Concentrations

Circulatory levels of the steroid hormones in study 1 patients were consistent with the stage of the simulated or extended menstrual cycle at the time of biopsy (Table 6). A significant reduction in serum progesterone concentrations was detected in premenstrual groups B (\*  $p < 0.01$ ) and C (\*\*  $p < 0.001$ ) when compared with control group A (LH+8-10). Serum oestradiol concentrations followed a similar pattern but did not reach significance. High serum concentrations of progesterone and oestradiol were detected in the hCG treated pseudopregnant group (D) and those with ectopic pregnancy (E), consistent with continuing endogenous steroid production.

**Table 7 : Serum Progesterone (nmol/l) and Oestradiol (pmol/l) Concentrations (Study 1)**

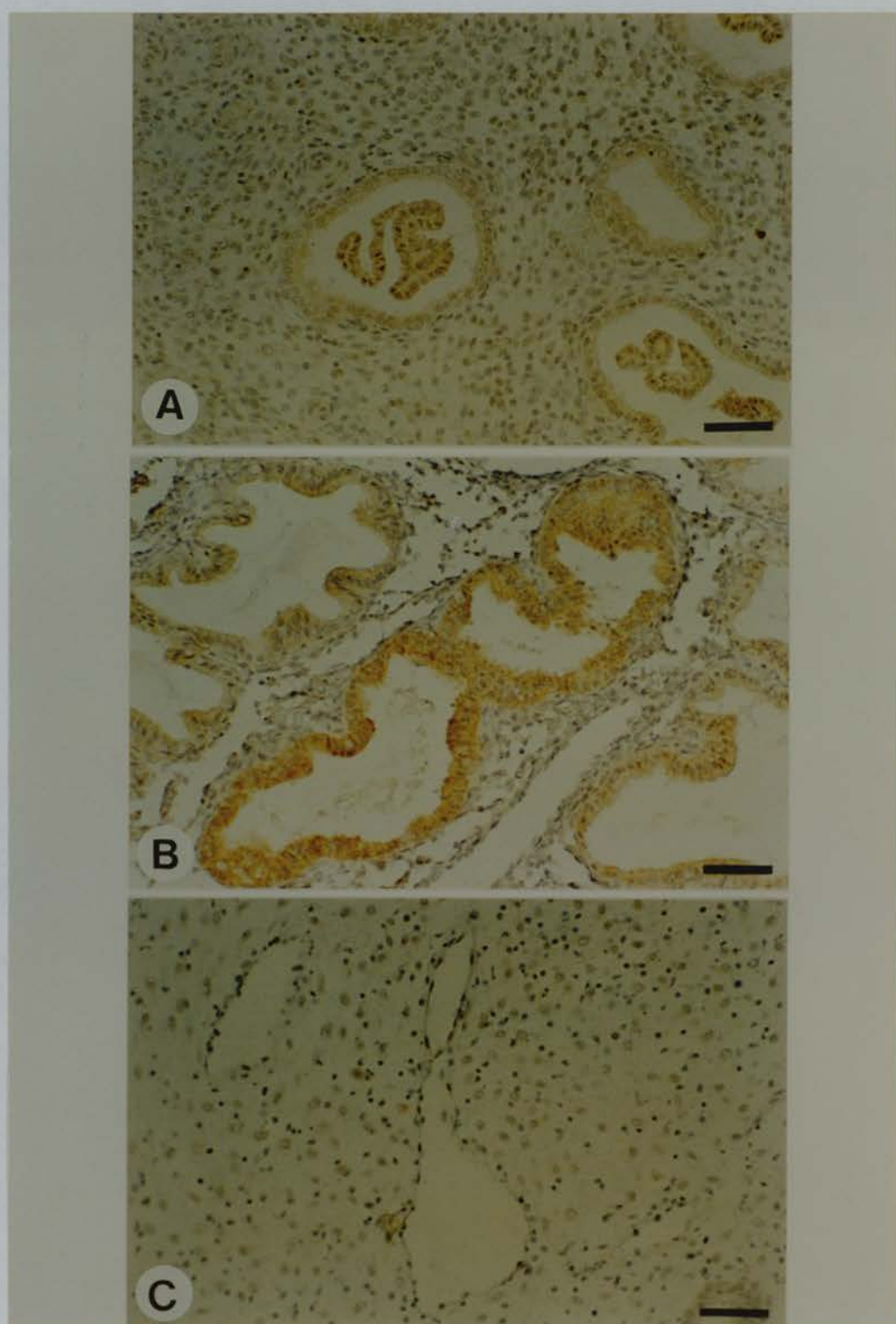
STUDY GROUP	PROGESTERONE		OESTRADIOL
	Mean $\pm$ SEM (Range)		Mean $\pm$ SEM (Range)
A	50 $\pm$ 11 (28.7 - 91.6)		454 $\pm$ 166 (234-1102)
B	11.3 $\pm$ 1.7 (8.7 - 17.6)	*	137 $\pm$ 49 (36 - 280)
C	3.5 $\pm$ 0.7 (2.4 - 6.4)	**	54 $\pm$ 7.5 (30 - 75)
D	70.1 $\pm$ 15.5 (28.3 - 112.3)		1386 $\pm$ 353 (801 - 2731)
E	34.6 $\pm$ 10.7 (11.9 - 60.7)		1496 $\pm$ 570 (683 - 3091)

**Figure 28** IL-8 immunoreactivity in endometrium collected from study 1 patients. Slight variations in immunostaining intensity and distribution were detected between the different groups. In the control group (A), IL-8 moderate immunoreactivity was limited to the perivascular cells. Withdrawal of progesterone triggered breakdown of the endometrium functionalis, and was associated with strong immunostaining for IL-8 in vessels and additionally in stromal and glandular components (B). Maintenance of progesterone produced lower levels of IL-8 immunostaining, although this was found to be lower in hCG induced pseudopregnancy (C) than in ectopic pregnancy (D).



**Figure 29** COX-2 immunoreactivity in endometrium collected from study 1 patients. Immunostaining for COX-2 was identical in distribution and intensity as that collected during the mid secretory phase (Chapter 3). An increase in immunostaining intensity in glandular epithelium was detected between 24 hours (A) and 48 hours (B) after withdrawal of exogenous progesterone. In particular, 48 hours after progesterone withdrawal, COX-2 immunostaining was reminiscent of that observed in menstrual phase endometrium (Chapter 3). Minimal immunostaining was detected when progesterone concentrations were maintained, either by administration of hCG or from ectopic pregnancy (C).



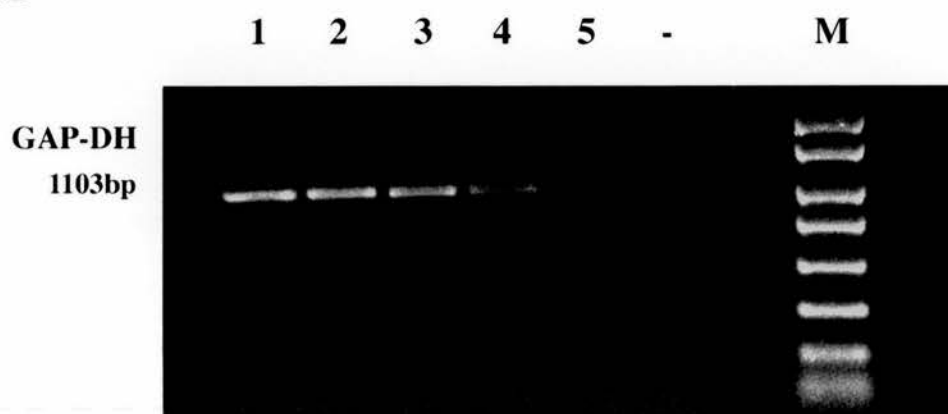
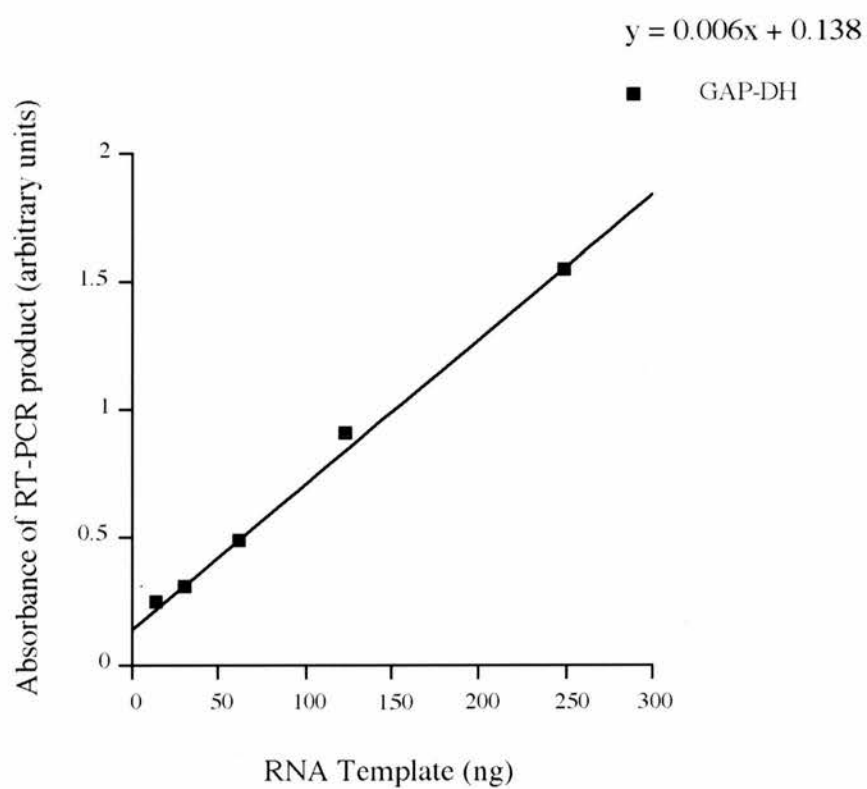


### Semi-Quantitative RT-PCR

GAP-DH RT-PCR and ELISA was repeated with correction of RNA amounts, producing signals (1103bp) of equivalent intensity for all samples (figure 31). The standard curve yielded the equation  $y = 0.006x + 0.138$  (figure 30). 60ng (+/- 10ng) of RNA was loaded into the RT-PCRs for IL-8, MCP-1 and COX-2. A single band was obtained for IL-8 (298bp), MCP-1 (210bp) and COX-2 (350bp) for the positive control RNA sample (QC). Distinct variations in signal intensity were observed between the samples from the different study groups (figure 31). Product yield was assessed by ELISA and expressed as the ratio of sample signal : QC signal.

A low signal was obtained from the control group (A) for IL-8, MCP-1 and COX-2. Withdrawal of progesterone resulted in an increase in mediator signal in the endometrium (groups B and C), which reached significance after 48 hours (C) for IL-8 ( $p < 0.01$ ) and COX-2 ( $p < 0.05$ ). Pharmacological rescue of the corpus luteum by hCG administration induced pseudo-pregnancy (D) and resulted in a reduction of IL-8 ( $p < 0.01$  compared to group C) and COX-2 mRNA concentrations, comparable with the control group (A). MCP-1 mRNA expression remained elevated. Similar expression levels were detected in decidua collected from cases of ectopic pregnancy (E), with a slight non-significant elevation in IL-8 mRNA levels.

**Figure 30** Standard curve for GAP-DH amplification. (A) Demonstration of the changes in signal intensity with varying standard RNA concentrations, by gel electrophoresis (250ng - 17.5ng). (B) After measurement of product yield by ELISA, the amount of RNA template included (x) was plotted against the absorbance of the GAP-DH RT-PCR product (y) at 450nm. This yields the equation  $y = 0.006x + 0.138$ , allowing calculation of RNA template concentrations. A linear relationship is achieved, indicating that the RT-PCR is not limited by reaction components and there is comparable efficiency for the different standards and samples.

**A****B**

**Figure 31** Expression of GAP-DH, IL-8, MCP-1 and COX-2 mRNA in endometrium collected from study 1 patients. Representative RT-PCR products from each study group are shown:

A = mid luteal, control group

B = 24 hours post progesterone withdrawal

C = 48 hours post progesterone withdrawal

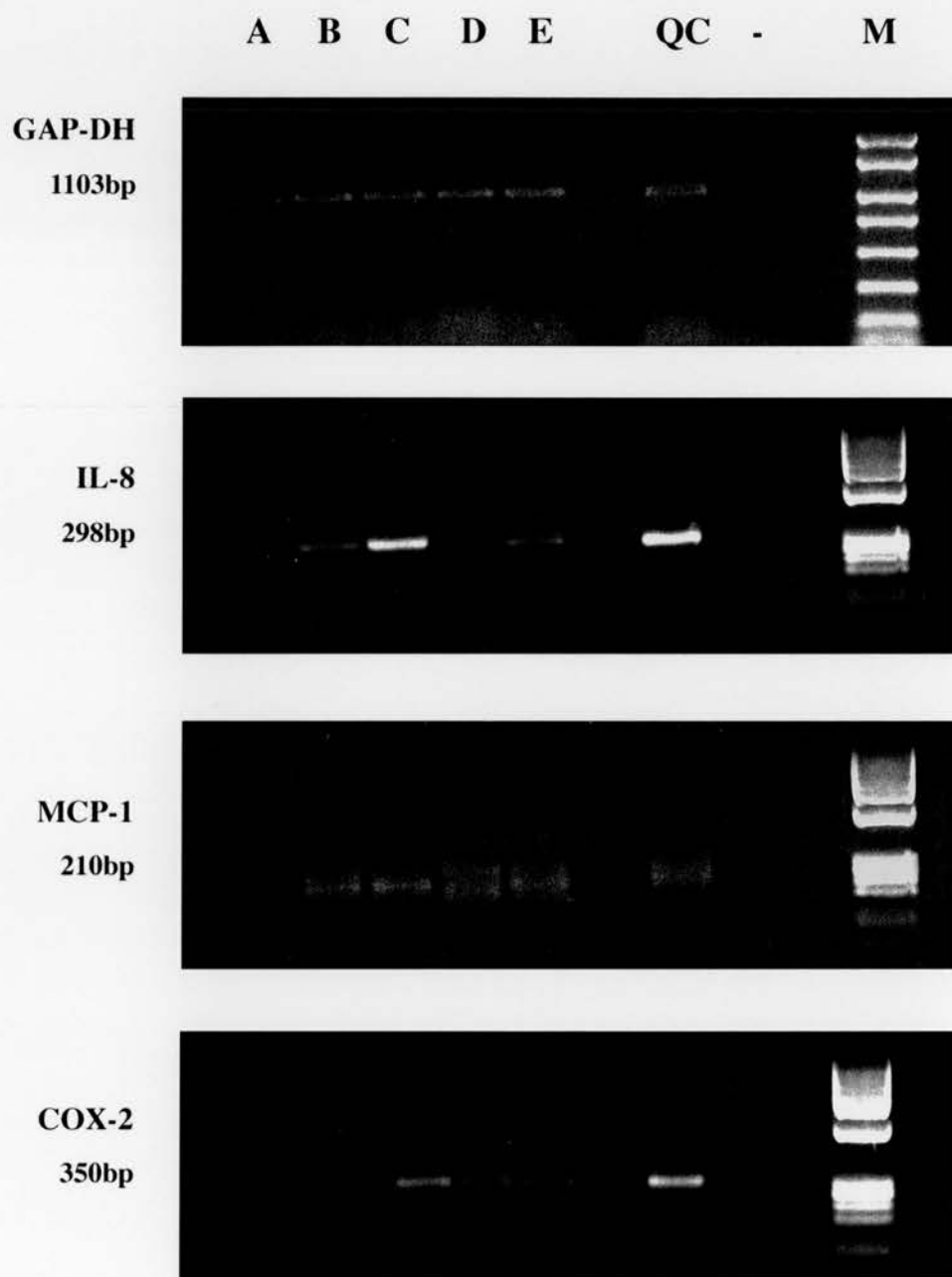
D = hCG maintained pseudopregnancy

E = ectopic pregnancy

QC = quality control

- = negative control

The upper panel indicates the equal loading of the RNA template into the subsequent mediator RT-PCRs. All bands for GAP-DH are of comparable intensity. The same RNA samples were subjected to RT-PCR for IL-8, MCP-1 and COX-2. For IL-8, no signal was detected in the control group. After withdrawal of progesterone, a strong signal of 298bp was present (B and C). Faint bands were detected in progesterone maintenance groups. A similar pattern was detectable for MCP-1, although signal s of comparable intensity were observed in the progesterone maintenance groups. No signal was detected for COX-2 in the control group, but a strong signal was identified from premenstrual phase endometrium (C). Faint bands were detectable in groups D and E.

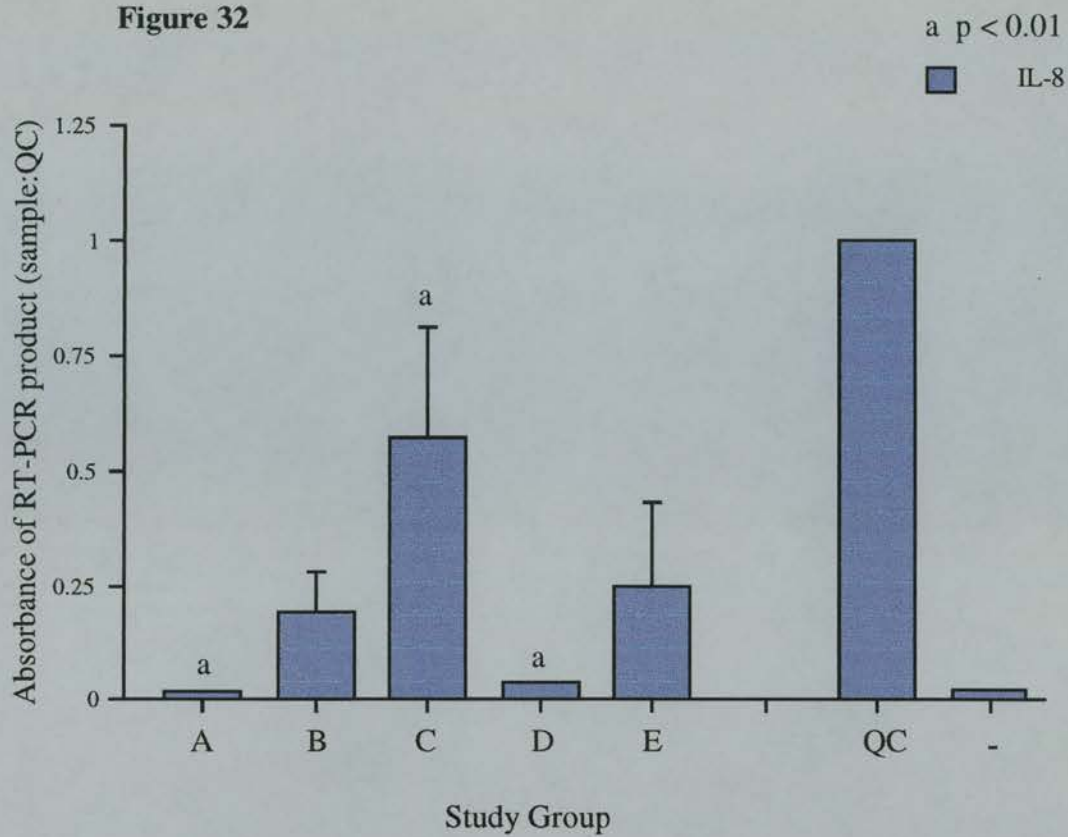




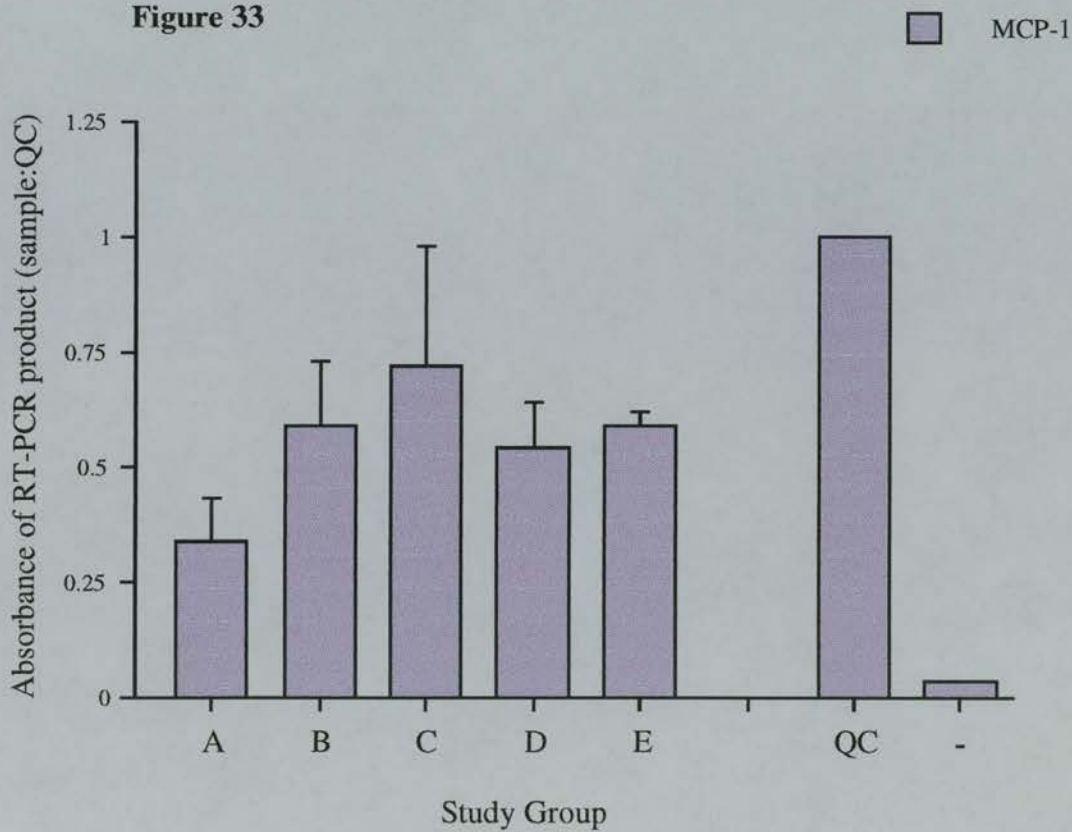
**Figure 32** IL-8 mRNA expression in study 1 endometrium. Low levels of expression of IL-8 were detected in the endometrium from the control group (A). The signal increased slightly 24 hours after progesterone withdrawal (B), and 48 hours after withdrawal (C) a significant elevation in mRNA transcript expression was detected ( $p < 0.01$ ) compared to the control group. In the pseudopregnant group (D) levels of IL-8 transcripts were low ( $p < 0.01$  to C group) and slightly higher levels were apparent in the endometrium collected from ectopic pregnancy. A strong signal was obtained from the quality control (QC) and no signal was detectable for the negative control.

**Figure 33** MCP-1 mRNA expression in study 1 endometrium. Variations were apparent in the levels of MCP-1 mRNA in the study groups (not significant). The lowest number of mRNA transcripts were detected in the control group (A), with an trend towards an increase following progesterone withdrawal (B and C). In the pseudopregnant (D) and ectopic (E) decidua, expression of MCP-1 remained elevated compared to mid secretory levels (A). A positive signal was achieved for quality control (QC) and no signal was obtained in the absence of RNA (-).

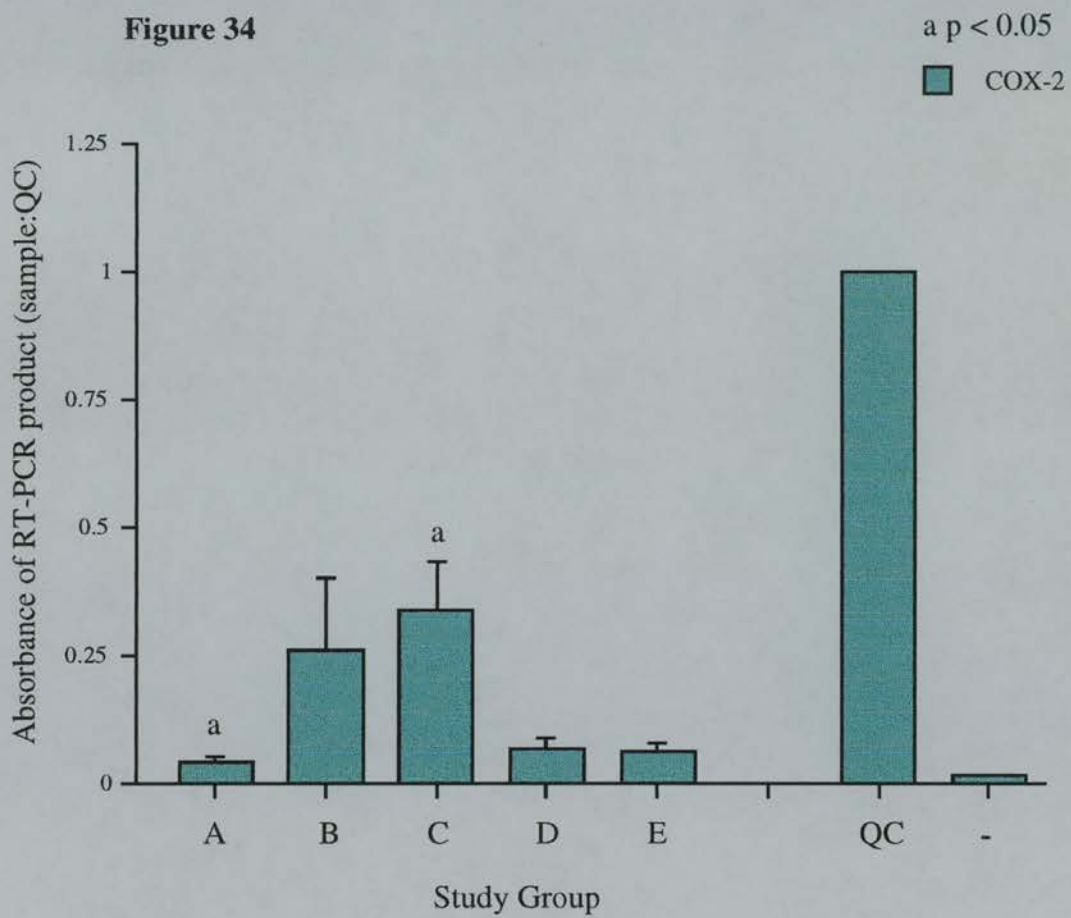
**Figure 32**



**Figure 33**

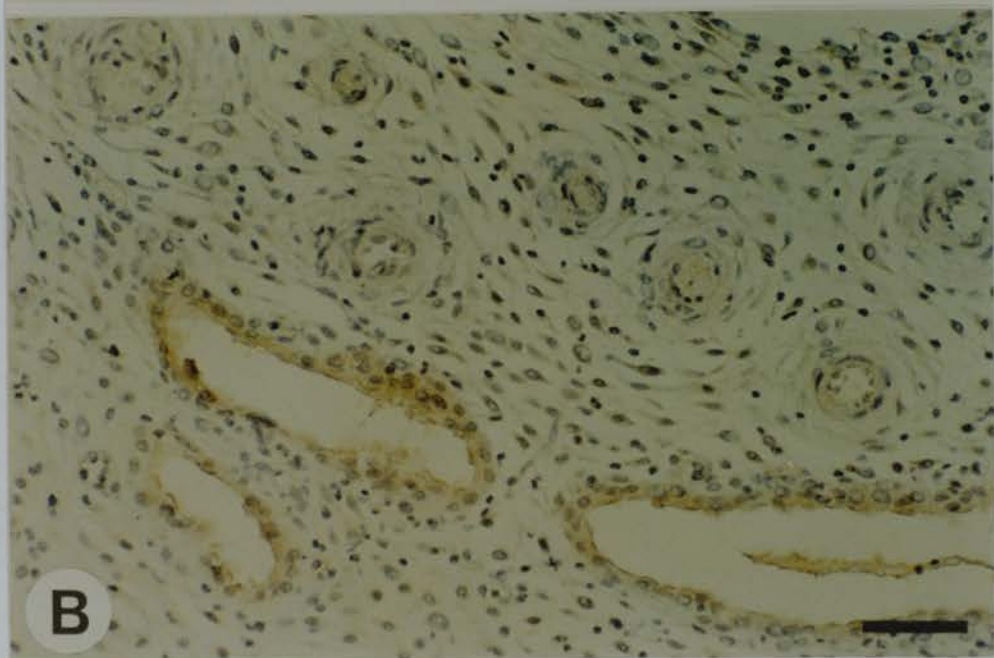
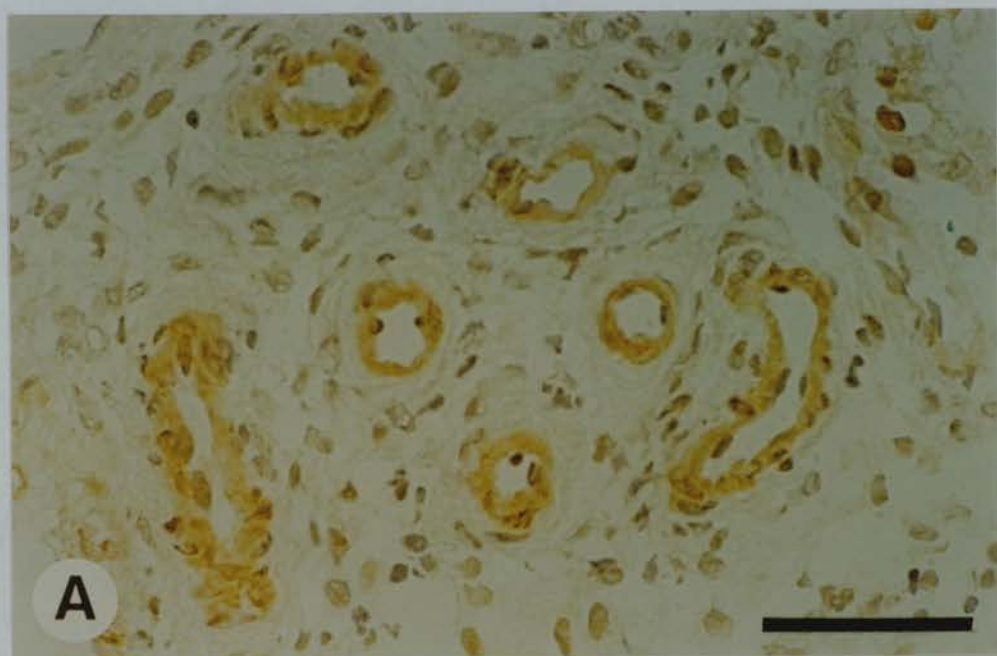


**Figure 34** A similar pattern of COX-2 expression levels was detected to IL-8 in the study patients. Very low levels of COX-2 mRNA were present in the control group (A), with a significant increase ( $p < 0.05$ ) observed 48 hours (C) but not 24 hours (B) after exogenous progesterone had been withdrawn. COX-2 mRNA expression in the pseudopregnant (D) and ectopic (E) pregnancies was consistent with those during the mid secretory phase (A). A strong signal was obtained from the quality control (QC) and no signal was detectable for the negative control (-).



Study 2      Immunohistochemical analysis of IL-8 and COX-2 expression in first trimester decidua collected at different time points following administration of RU486 was examined (figure 35). No significant differences were detectable in the intensity or distribution of either IL-8 or COX-2 immunoreactivity, although a slight elevation in COX-2 immunostaining was apparent both 6 and 12 hours after antigestogen administration, compared to the control group (figure 36). COX-2 immunoreactivity was predominantly localised to the glandular epithelium, and marked variations in staining intensity were observed within the study groups.

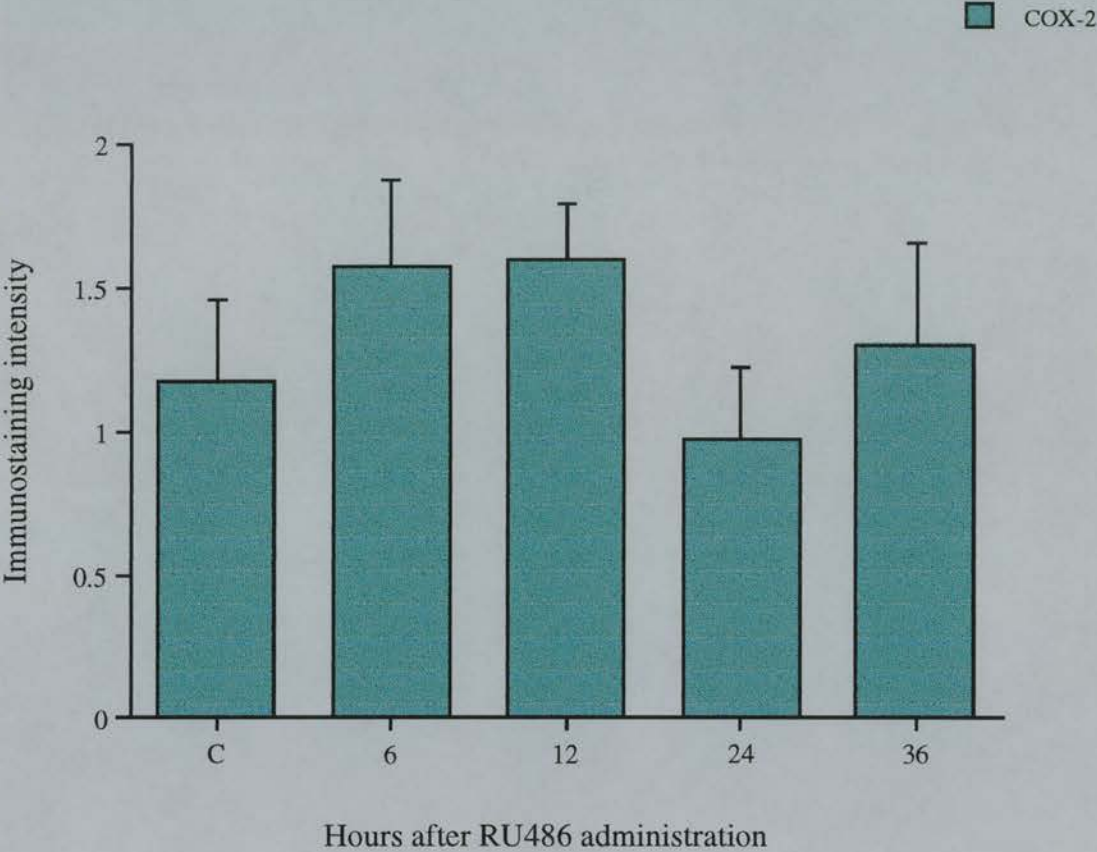
**Figure 35** Immunostaining for IL-8 (A) and COX-2 (B) in first trimester decidua following pharmacological withdrawal of progesterone (study 2). No distinct changes in IL-8 or COX-2 immunoreactivity were detected at the different time points following antigestogen administration. IL-8 immunostaining was apparent in the perivascular cells in all tissues examined, while COX-2 immunostaining was primarily localised to the glandular epithelium. Scale bars = 50µm.





**Figure 36** Histogram illustrating COX-2 immunostaining in decidual samples collected at different time points following antigestogen administration (Study 2). A slight transient increase in the degree of COX-2 glandular immunoreactivity was detected 6 and 12 hours after pharmacological withdrawal of progesterone when compared to control group (C), with reduced intensity of staining detected after 24 hours.

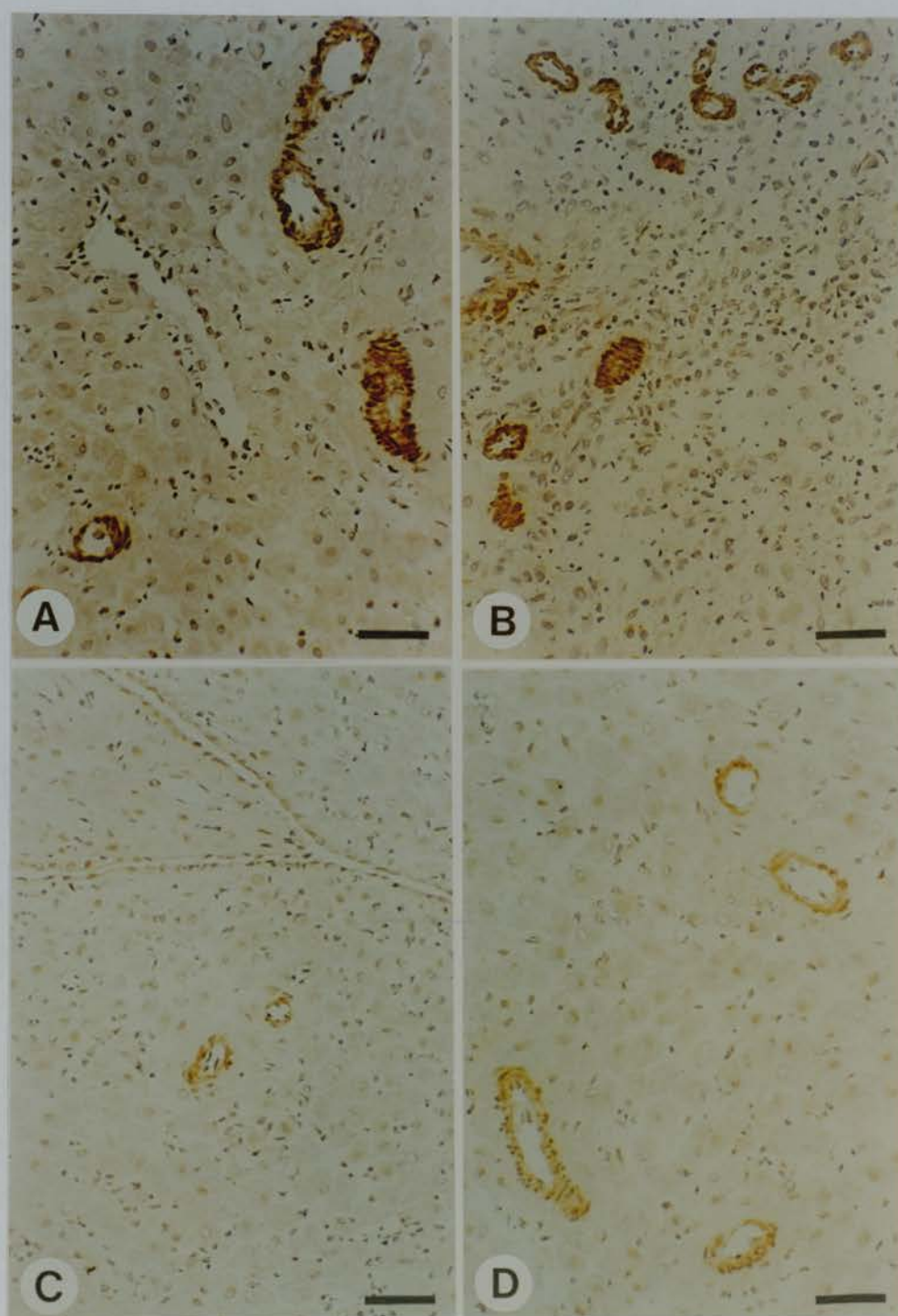
Figure 36



Study 3      Preinsertion biopsies were histologically consistent with the stage of cycle calculated from last menstrual period (LMP). Widespread morphological changes were observed after insertion of the Lng-IUS, most notably extensive decidualisation of stromal cells and glandular atrophy. Once the Lng-IUS was *in situ*, the histological appearances of the endometrium in the proliferative and secretory phase were indistinguishable, thus sample data at each of the post-insertion stages (1, 3, 6 and 12 months post-insertion) was pooled.

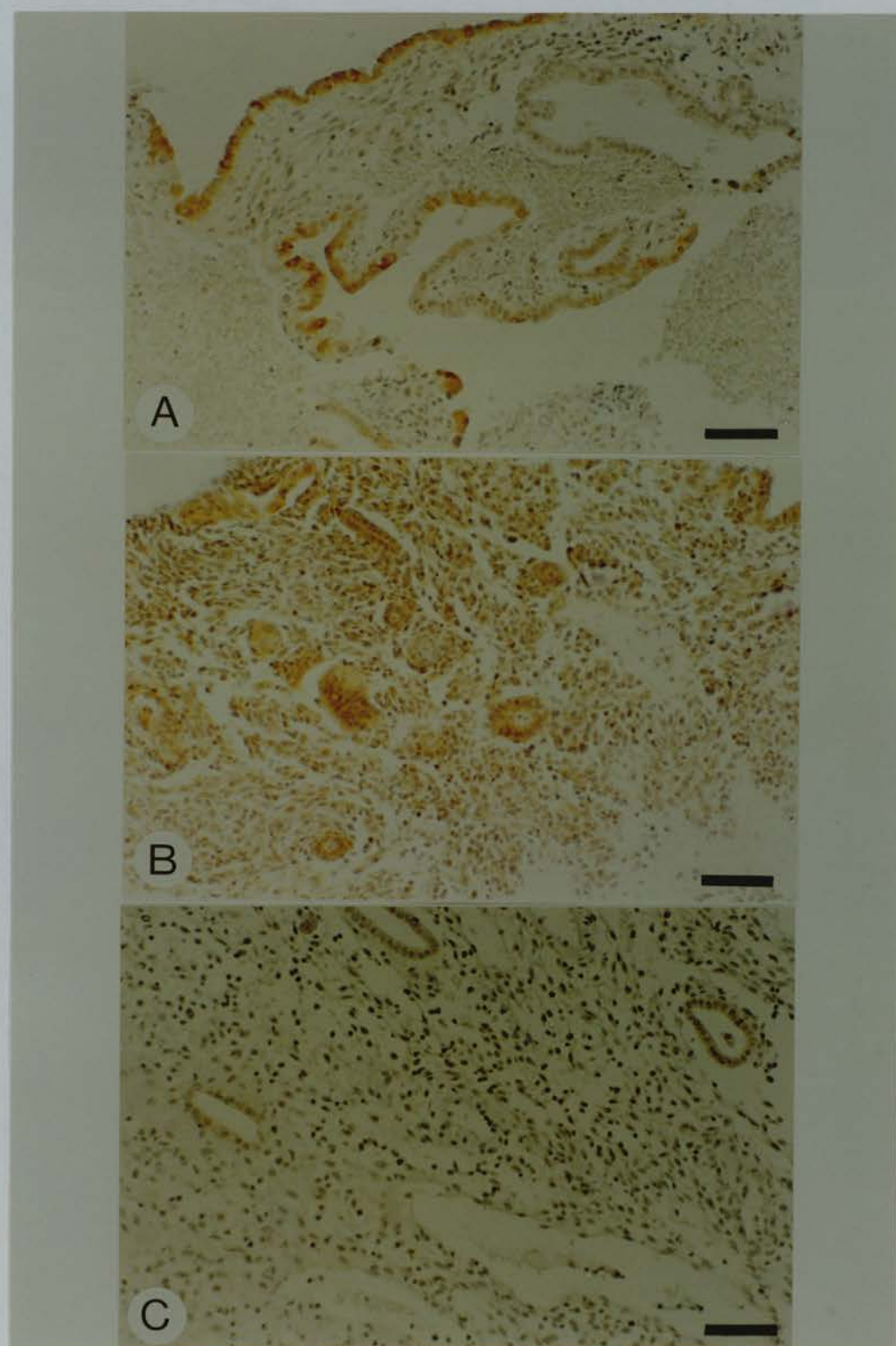
IL-8 immunoreactivity was detected around spiral arterioles and other blood vessel types (figures 37 and 39). A decrease in IL-8 immunostaining was apparent at 3 and 6 months postinsertion. COX-2 immunoreactivity in preinsertion biopsies was consistent with the stage of the menstrual cycle (figures 38 and 40). High levels of immunostaining were detected one month post insertion, comparable to premenstrual expression of COX-2. After 3, 6 and 12 months of exposure to Lng, a slight reduction in COX-2 immunoreactivity was observed, although marked patient to patient variations persisted.

**Figure 37** IL-8 immunoreactivity in endometrium exposed to intrauterine levonorgestrel via an Lng-IUS. (A) Very intense perivascular immunostaining for IL-8 was detected one month after insertion of the Lng-IUS. (B) Immunoreactivity was slightly reduced after 3 months exposure, and (C & D) a further decline in the degree of staining was apparent after 6 months exposure to local synthetic progestin. Scale bars = 50µm.



**Figure 38** COX-2 immunoreactivity in endometrium exposed to intrauterine levonorgestrel via an Lng-IUS. Strong immunostaining for COX-2 was detected one month after insertion of the Lng-IUS, in the glandular epithelium (A) and in the glandular epithelium, vasculature and stroma (B). Markedly reduced immunoreactivity was detected after 6 months exposure to Lng (C). Scale bars = 50µm.







**Figure 39** IL-8 immunoreactivity before and after insertion of a Lng-IUS. Strong perivascular immunostaining was detected for IL-8 in tissues collected prior to insertion of the device (separated into proliferative and secretory phases). Intense immunostaining was detected in endometrial biopsies collected 1 month after insertion of the Lng-IUS, but a marked reduction was evident after 3 months exposure to local Lng, with a further reduction 6 months post-insertion. Insufficient tissues were collected in frozen blocks to assess IL-8 immunostaining at 12 months postinsertion, due to the atrophic nature of the endometrium.

**Figure 40** COX-2 glandular immunoreactivity in endometrial biopsies collected before and after insertion of a Lng-releasing IUS. Preinsertion biopsies displayed patterns of immunostaining characteristic of the stage of the menstrual cycle, with strongest glandular immunostaining in the menstrual and premenstrual phases. Immunoreactivity comparable to that observed in the premenstrual biopsies persisted at 1 and 3 months postinsertion of the Lng-IUS. After 6 and 12 months of exposure, the degree of COX-2 immunostaining was reduced.

Figure 39

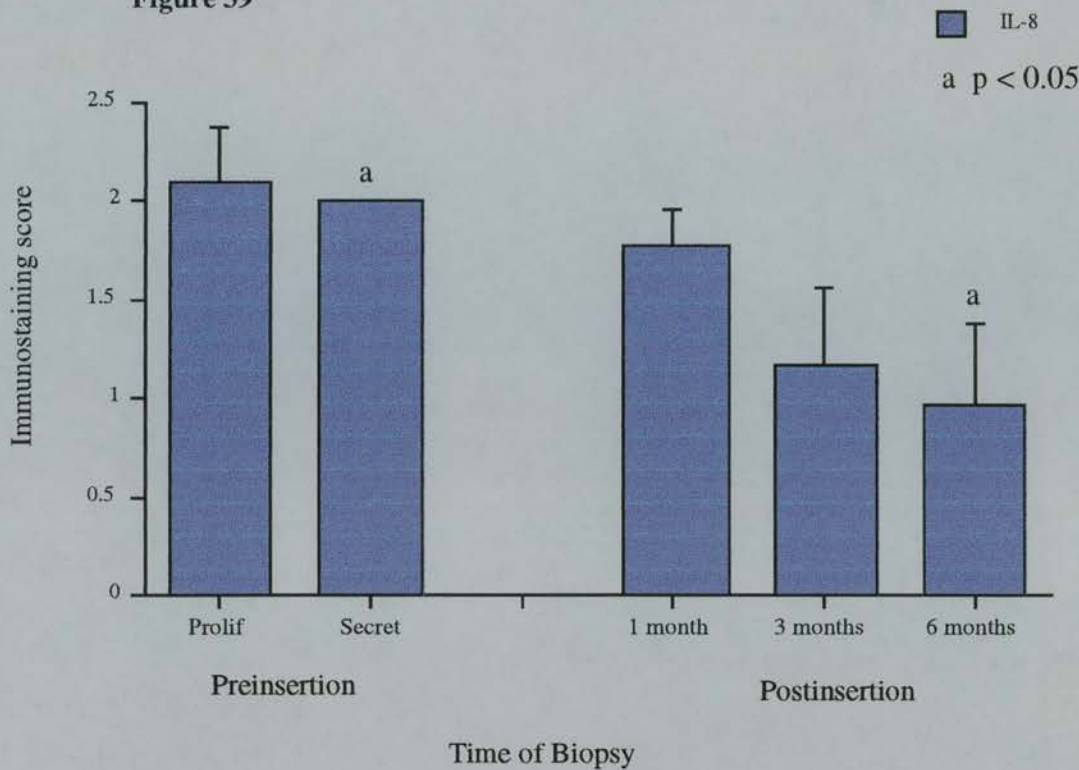
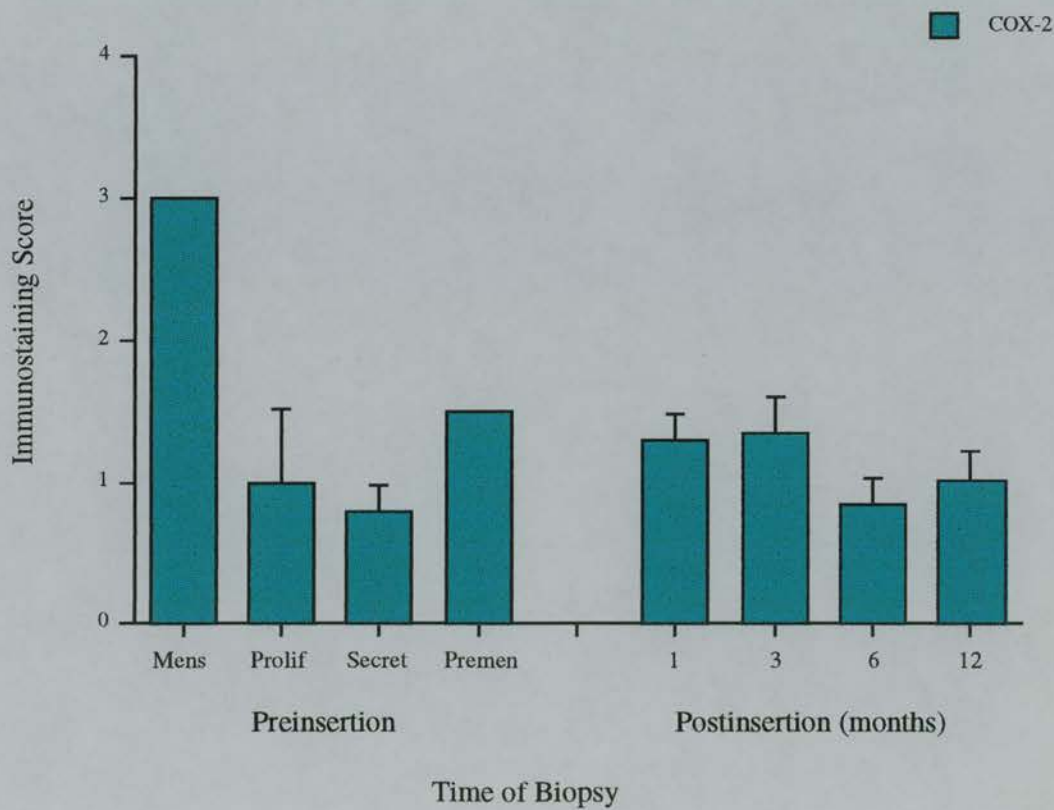


Figure 40



## 4.4 Discussion

The current chapter describes the effect on endometrial inflammatory mediator expression by *in vivo* perturbation of the progesterone environment. Three separate and distinct methods of progesterone modulation were utilised to examine the regulation of IL-8, MCP-1 and COX-2.

### Study 1

Study 1 was designed to clarify previous findings (chapter 3) of inflammatory mediator expression in normal non-pregnant and pregnant endometrium. The premenstrual period was artificially simulated by administration and withdrawal of exogenous progesterone at timed intervals before tissue biopsy, to examine events associated with the onset of menses. Local endometrial events taking place in very early pregnancy were studied by administration of hCG in incremental doses to mimic natural production of gonadotrophin by the embryo. This "rescues" the corpus luteum, allowing continuation of endogenous progesterone production and induces a state of pseudopregnancy. Finally, decidua from women with a pregnancy ectopic to the uterus was collected to observed the differences in mediator expression without the presence of intrauterine trophoblast.

Histological features of tissues from each study group were consistent with the date of the menstrual cycle and with the prevailing steroid environment. The number and subtype of leukocytes in the endometrium were examined by Critchley et al (1998c). CD56+ LGLs formed the major constituent of leukocytes present, with high numbers of stromal macrophages. Few neutrophils were detectable. This is compatible with the fact that our biopsies were collected prior to the reported neutrophil infiltrate in the immediate premenstrual phase (Poropatich et al., 1987). Sex steroid distribution was also examined and the expression of ER and PgR (A and B forms) was found to be consistent with published literature for the normal menstrual cycle (Lessey et al., 1988; Wang et al., 1997b) (see introduction, section 1.2). This is confirmation of the reliability of this study design as a simulation of menstruation.

The significant elevation in IL-8 and COX-2 mRNA expression levels premenstrually (B and C), and low numbers of IL-8 and COX-2 mRNA transcripts in both pregnant groups (D and E) are in agreement with data from the normal cycle endometrium (chapter 3). Co-workers examined chemokine secretion from study 1

endometrial explants during a 24 hour culture period by ELISA. Elevations in IL-8 ( $p < 0.05$  compared with control group A) and MCP-1 (not significant) production from premenstrual endometrium, collected 48 hours post progesterone withdrawal were reported. The production of chemokines is therefore compatible with the increased mRNA expression levels observed in the present study. Examination of PG production by the endometrial explants confirmed that the COX-2 mRNA detected is being translated and is responsible for elevated PG production premenstrually. In particular, PGF<sub>2</sub> $\alpha$  production was significantly increased both 24 and 48 hours following progesterone withdrawal compared to control group A, and markedly reduced when pseudopregnancy was induced (D).

These data provide supporting evidence for the downregulation of IL-8 and COX-2 by progesterone, and for their proposed involvement in the initiation of menstruation. Failure to detect a significant rise in MCP-1 expression may indicate differences in the regulatory effect of progesterone on this chemokine. Higher levels of expression were observed in the mid secretory (A) and pregnant (D and E) groups, which suggests that progesterone inhibits MCP-1 expression to a lesser extent than IL-8. First trimester decidua examined for MCP-1 immunoreactivity (Chapter 3, section 3.3) also exhibited relatively high levels of immunostaining. Several differences in the regulation of the chemokines IL-8 and MCP-1 have previously been reported, including stimulation of MCP-1 by PDGF, IFN- $\gamma$ , PRL and LIF (Yoshimura et al., 1990; Seitz et al., 1994; Townson et al., 1996; Hartner et al., 1997). Furthermore, MCP-1 has been demonstrated to be induced preferentially by Th2 cytokines (Rollins et al., 1991; Warmington et al., 1996; Gu et al., 1997), which are believed to be predominant in early pregnancy (Wegmann et al., 1993). This is in contrast to IL-8, whose expression is generally upregulated in response to Th1 inflammatory cytokines, and is negatively regulated by the Th2 cytokine IL-10 (Kunkel, 1996). A delicate balance of regulatory factors is likely to exist in the uterus to carefully modulate the expression of local inflammatory mediators. It is interesting that the higher levels of MCP-1 expression in the early pregnant uterus correlate with the continued increase in the numbers of LGLs and macrophages in the decidual stroma (Bulmer et al., 1988; Loke et al., 1997). In support of this, a recent publication strongly supports a relationship between MCP-1 expression and macrophage recruitment in the peri-implantation mouse uterus (Wood et al., 1997).

## Study 2

The effect on decidual mediator expression following the pharmacological

withdrawal of progesterone by RU486 administration was examined in study 2. No distinct changes in IL-8 immunoreactive protein were detected at any of the time points following antigestogen administration. However, when chemokine secretion by decidua cultured for 24 hours was measured by ELISA, a significant increase in IL-8 production was detected 6 hours after antigestogen administration ( $p < 0.019$ ) with respect to the control group (I). A non-significant increase in MCP-1 was observed 6 hours post RU486 administration, followed by a significant decrease between 6 and 12 hours after mifepristone (Critchley et al., 1996). This method is likely to be more effective at detecting transient variations in expression levels than immunohistochemistry.

COX-2 immunoreactivity was examined in decidua collected at each of the time points following antigestogen administration. Although no significant differences were detected in the degree of immunostaining between study groups, there did appear to be a trend for elevated COX-2 expression 6 and 12 hours after RU486 administration. In addition, distinct variations in COX-2 immunostaining were observed between subjects, reminiscent of variations detected in first trimester decidua (Chapter 3, 3.3). This problem is likely to be due to the heterogeneous nature of this tissue, with incomplete decidualisation resulting in areas of fully decidualised endometrium whilst other regions maintain secretory type characteristics.

Co-workers investigated the effect of progesterone withdrawal on leukocyte subpopulations in first trimester decidua, and reported a significant elevation on the numbers of macrophages 24 hours after RU486 administration (Critchley et al., 1996). No significant changes in other subpopulations were reported but LGL numbers appeared to fall over the time course and by 36 hours a non-significant increase in neutrophil numbers was observed. These features are consistent with the inflammatory type response associated with progesterone withdrawal, either premenstrually or in early pregnancy. It is important to note that progesterone withdrawal by pharmacological blockade of the receptor is likely to be a more rapid process than the physiological withdrawal premenstrually. This may explain differences in leukocyte populations and chemokine dynamics between studies 1 and 2.

### **Study 3**

The effect of uterine progestin delivery, via a Lng-IUS, on local mediator expression



was examined. Whilst proving to be an effective long-term contraceptive and treatment for menorrhagia, this mode of intrauterine contraception is flawed by the common side effect of breakthrough bleeding experienced by the majority of users (Sivin et al., 1983; Odland et al., 1990). Considering the proposed involvement of the chemokines and PGs in normal menstruation, these are potential candidate mediators for investigation into the bleeding episodes caused by Lng modulation of the endometrium.

Following insertion of the Lng-IUS, the endometrium becomes markedly decidualised and atrophic (Silverberg et al., 1986). Glandular activity is diminished and vessels appear distended. Leukocyte infiltrate into the endometrium is augmented by the presence of an IUD (Sheppard, 1987) and macrophages in particular become adherent to the device and are a potential source of PGs (Myatt et al., 1975). Characterisation of leukocyte subtypes in the Lng-IUS endometrium by colleagues revealed the presence of high numbers of CD56+ LGLs and macrophages, whilst few neutrophils were identifiable. A significant increase in the numbers of macrophages was identified one month after insertion of the device (Critchley et al., 1998b). Increased numbers of uterine macrophages have been correlated with bleeding disorders in Norplant users (Clark et al., 1996). Whilst endometrial MCP-1 was not examined in the present study, IL-8 and COX-2 immunoreactivity remained high one month after insertion of the Lng-IUS, then was reduced at 3 and 6 months postinsertion. It was not possible to examine adequate numbers of biopsies collected 12 months after insertion of the Lng-IUS for IL-8, due to the atrophic nature of the endometrium by this stage. Similarly, insufficient endometrium could be collected to extract RNA for RT-PCR studies.

Examination of the steroid receptor content of these tissues has revealed that after one month of intrauterine exposure to Lng, both ER and PgR are absent in glandular epithelium and markedly reduced in stromal compartment of the endometrium (Critchley et al., 1998a). This pattern continues for approximately 6 months, after which a gradual, but significant return of PgR in epithelial glands is observed. Immunohistochemical studies indicate that the few PgR remaining in the stroma are likely to be PgR<sub>A</sub> (Critchley et al., 1998a), and the return of glandular PgR<sub>A</sub> between 6 and 12 months correlates to the restoration of functional activity (as defined by *in vitro* activity and immunoreactivity of progesterone sensitive PGDH) (Critchley et al., 1998a). This is consistent with the expression of the subtypes in the normal menstrual cycle and first trimester decidua, with a preferential down regulation of PgR<sub>B</sub> by progesterone in the secretory phase and early pregnancy

(Wang et al., 1998).

The significance of differential PgR expression in the endometrium is not understood. The predominance of PgR<sub>A</sub> when decidualisation of the endometrium takes place, both in the normal menstrual cycle and in Lng exposed endometrium, would suggest that this isoform has a role in mediating the transformation. Furthermore, from the temporal expression during the menstrual cycle, it would seem likely that the inhibitory effect of progesterone on chemokine and COX-2 expression is mediated via PgR<sub>A</sub>.

We have hypothesised that the downregulation of PgR (both A and B subtypes) by local levonorgestrel delivery could be involved in the cascade of events which results in breakthrough bleeding, especially as an adjustment in uterine bleeding patterns seems to accompany the increased expression of the steroid receptors between 6 and 12 months of use. Progesterone sensitive chemokines and COX-2 could conceivably have a role in such bleeding episodes. This is reinforced by the strong immunoreactivity for both IL-8 and COX-2 after insertion of the Lng-IUS and subsequent reduction with continued Lng exposure and the return of PgR. This argument is, however, complicated by findings from women using subcutaneous Norplant implants, which release an identical progestin into the peripheral circulation. Norplant users also experience breakthrough bleeding, yet intense immunoreactivity for PgR and ER is detectable in the endometrium throughout the menstrual cycle in these subjects (Critchley et al., 1993). A slight improvement in bleeding patterns was reported for women using Norplant following administration of a single postmenstrual dose of RU486 (Wang et al., 1997a). This might suggest that a different mechanism is operating depending on the method of levonorgestrel administration.

The above studies all involved the modulation of the local progesterone environment, allowing further investigation into the role of this steroid in regulating local factor expression *in vivo*. Analysis of endometrial mRNA expression of IL-8, MCP-1 and COX-2 by RT-PCR following withdrawal of exogenous progesterone lends support to the hypothesis of a regulatory influence of progesterone on the mediators. Pharmacological withdrawal by RU486 administration did not produce such clear results as far as mediator expression was concerned, as mRNA levels could not be measured. The Lng-IUS provides a complex system of progesterone manipulation, and the involvement of chemokines and PGs in the breakthrough bleeding episodes is reinforced by the strong expression of IL-8 and COX-2 after



insertion of the device. The mechanisms involved in their regulation in this environment are as yet not understood.

## **CHAPTER 5**

### **EXAMINATION OF INFLAMMATORY MEDIATOR EXPRESSION USING AN *IN VITRO* CULTURE SYSTEM**

## 5.1 Introduction

The previous two chapter's findings support the hypothesis that progesterone has a regulatory role in the expression of inflammatory mediator expression. In this chapter, the potential modulation is further investigated by employing an *in vitro* culture system. In addition, the stimulation and induction of the chemokines and COX-2 is examined. Much has been learnt about the regulation of chemokine and prostaglandin production by studying the effects of inflammatory mediators, mitogens, growth factors and anti-inflammatory agents *in vitro*. Some investigation into the mechanisms involved in induction and inhibition of expression has been conducted. This provides a greater understanding of the expression of local mediators and their complex interactions *in vivo*.

IL-8 and MCP-1 are produced and secreted by a number of cell types, including fibroblasts, endothelial and epithelial cells, monocytes and other lymphoid cell types (Baggiolini et al., 1997). Pro-inflammatory mediators, such as IL-1 and TNF- $\alpha$ , are potent stimulators of IL-8 and MCP-1. A rapid, but transient, induction of mRNA and protein levels in response to stimuli has been reported by a number of independent researchers. Using isolated vascular endothelial cells, it was demonstrated that TNF- $\alpha$ , LPS and IL-1 $\beta$  all stimulated elevated expression of the 1.8 kb IL-8 mRNA (Strieter et al., 1989). An induction was apparent after one hour, with maximal expression by 4 - 8 hours. This was reinforced by *in situ* hybridisation for mRNA in isolated endothelial cells, before and after treatment with TNF- $\alpha$  (Strieter et al., 1989). Similar findings were obtained by Brown and colleagues with induction of IL-8 and MCP-1 mRNA from microvascular endothelial cells following treatment with TNF- $\alpha$ , LPS and IL-1 $\beta$  (Brown et al, 1994). Interestingly, differences in the potency of the stimulants were detected for IL-8 and MCP-1, with maximal stimulation of MCP-1 by IL-1 and IL-8 by TNF- $\alpha$ . Further regulatory differences have been reported, particularly the induction of MCP-1 by PDGF and IFN- $\gamma$ , neither of which have any effect on IL-8 expression (Yoshimura et al., 1990; Brown et al., 1994). It has been concluded that the upstream regulatory regions for both chemokines must differ significantly, although no differences have been recognised in the putative transcriptional regulatory elements (Mukaida et al., 1989; Shyy et al., 1990).

A recent investigation into vascular smooth muscle cells derived from rat kidney reported the stimulation of MCP-1 mRNA with the cytokine LIF (Hartner et al.,

1997). This is of prime interest for uterine MCP-1 expression, as LIF appears to play a vital role in the process of implantation (Chen et al., 1995a) (see introduction, section 1.14), and is abundant in the decidualised endometrium. The same study examined the role of IL-6, a pro-inflammatory mediator closely related to IL-1 and TNF- $\alpha$  (Hartner et al., 1997). A transient increase in MCP-1 expression was observed following exposure to IL-6 for 2 hours. The validity of these *in vitro* data was confirmed by a recent investigation, using gene knockout technology to eliminate IL-6 function *in vivo*. The IL-6 receptor deficient mice had a reduced capacity to mount an inflammatory response (Romano et al., 1997), and in particular, a distinct reduction in chemokine production (IL-8 and MCP-1) was detected.

Detailed investigation into endometrial expression of IL-8 and MCP-1 has been conducted. Isolated stromal and glandular cells were cultured with IL-1, TNF- $\alpha$  (Arici et al., 1993) and TGF- $\beta$  (Arici et al., 1996). Marked elevations in IL-8 mRNA and protein were observed, with synergistic increases with combinations of IL-1 and TNF- $\alpha$ , TGF- $\beta$  and IL-1, and fetal bovine serum (FBS) with IL-1. These effects were dose dependent, and culturing for various lengths of time indicated that maximal induction occurred by 3 - 6 hours, with a gradual decline back to basal levels after 6 hours. Similar examination into the regulation of MCP-1 expression confirmed previous studies on non-reproductive tissues, reporting stimulation with IL-1, TNF- $\alpha$ , IFN- $\gamma$  (Arici et al., 1995) and FBS (Kovacs et al., 1996).

The effect of steroids on chemokine expression has previously been examined. A reduction in the secretion of immunoreactive IL-8 from chorio-decidual and endometrial cells following a 24 hour incubation with progesterone was described by Kelly et al, and a similar inhibition was observed for the synthetic progestin MPA and glucocorticoid dexamethasone (Kelly et al., 1994). This reveals a potential mechanism for the finding reported by Sinosich et al, that exposure to RU486 results in stimulation of neutrophil influx into placenta in monkeys (Sinosich et al., 1989). A similar suppression of IL-8 production occurs in uterine-cervical cells, which can be reversed by treatment with antigestogen RU486 (Ito et al., 1994). The effect of oestradiol on IL-8 expression has not been examined, but MCP-1 expression in macrophages and fibroblasts was found to be markedly downregulated by the steroid (Kovacs et al., 1993; Kovacs et al., 1996). In ovarian tissue collected from women being treated for IVF, an elevation in MCP-1 protein levels following hCG administration was reported (Arici et al., 1997). Treatment with oestradiol and progesterone did not alter MCP-1 levels, although a previous study by the same authors reported decreased levels of MCP-1 mRNA in endometrium biopsied from

women taking oral progestin (Arici et al., 1995). Evidence from our own laboratory would support a downregulation of MCP-1 by progesterone in chorio-decidual cells (Kelly et al., 1997b).

Prostaglandin production by the utero-placental unit must be under strict regulation to prevent inappropriate stimulation of an inflammatory response. PGs are key mediators in many events necessary for pregnancy, including roles in promoting decidual cell formation (Kennedy, 1986) and vascular permeability (Kennedy et al., 1981) at the time of implantation. However, PGs have also been implicated in the initiation of menstruation and parturition (Kennedy et al., 1981; Baird et al., 1996). The regulation of PG production has been examined in many culture systems.

Stromal cells isolated from first trimester decidua produce elevated levels of PGE<sub>2</sub> and PGF<sub>2α</sub> in response to IL-1β stimulation (Ishihara et al., 1992). A significant increase is detectable after 8 hours in culture, and corresponds to an elevation in immunoreactive COX-2 enzyme (Ishihara et al., 1995). The same is true for endometrial glandular and stromal cells, with significant elevation in PG production following stimulation with IL-1 and TNF-α (Chen et al., 1995). Phorbol esters, including PMA, cause a dose dependent increase in PG production, which is augmented by IL-1β (Cole et al., 1995). Research into dynamics of vascular endothelial cell COX-2 expression reveals that a marked increase in COX-2 mRNA is detectable within 2 hours culture in the presence of serum (Pritchard Jnr et al., 1994). A subsequent decline in mRNA was observed after 6 hours, in line with the known rapid degradation of the transcripts. The elevated expression of COX-2 mRNA was correlated with a maximal increase in COX-2 enzyme and secreted PGE<sub>2</sub> after 8 hours.

Before the discovery of the second isoform of COX (COX-2), several independent studies revealed a regulatory role of PG production by ovarian sex steroids (Blatchely et al., 1974; Castracane et al., 1975). Isolated endometrial glands in the proliferative and secretory phases produce less PGE<sub>2</sub> and PGF<sub>2α</sub> following culture for 24 hours with progesterone (Abel et al., 1980), and withdrawal of progesterone through RU486 administration stimulates PG synthesis from endometrium and decidua (Kelly et al., 1986; Smith et al., 1987). Recent research has attributed the downregulation of PG production by glucocorticoids or progesterone to an inhibition of COX-2 (Kelly et al., 1986; Smith et al., 1987; O'Banion et al., 1991; Kujubu et al., 1992; Mitchell et al., 1994). Dexamethasone has no effect on COX-1 synthesis or activity (O'Banion et al., 1992). This conclusion has been confirmed in reproductive



tissues. *In vitro* culture of endometrial stromal cells with dexamethasone caused an inhibition of COX-2, whilst COX-1 remained unaffected (Jacobs et al., 1994). In cultured decidual stromal cells dexamethasone and progesterone inhibited IL-1 $\beta$  stimulated PG production by 50% (Ishihara et al., 1995), and this again was reflected by a decrease in COX-2 expression. PMA and other stimulants, serum and IL-1, stimulate PG production through increased expression of COX-2 (Kubjuba et al., 1991; O'Banion et al., 1991; Xie et al., 1991). Negligible stimulation of COX-1 occurs (DeWitt, 1991; Simmons et al., 1991; O'Banion et al., 1992; Pritchard Jnr et al., 1994). This finding reinforces the role of COX-2 as an inflammatory mediator and COX-1 in the constitutive production of PGs (Simmons et al., 1991; Vane et al., 1994).

In the present study the primary response of IL-8, MCP-1 and COX-2 mRNA expression by endometrial and decidual explants was examined after treatment with pro-inflammatory stimulants, phorbol myristate acetate (PMA) and FCS, and progesterone. PMA, a pharmacological activator of protein kinase C, stimulates all three inflammatory mediators with similar kinetics to IL-1 or TNF- $\alpha$  (Mukaida et al., 1990). Detailed studies of the time course of PMA induction of MCP-1 and COX-2 revealed a rapid but transient elevation in mRNA expression, typically with a signal detectable after 30 minutes, maximal after 2 - 4 hours then reduced to basal levels after 16 - 24 hours, consistent with the role of these agents in acute inflammation (Shyy et al., 1990; Kubjuba et al., 1992). PMA is known to interact with phorbol ester response elements (TRE) in the upstream region of the genes (Shyy et al., 1990) and acts, at least in part, to stabilise mRNA transcripts (Ristimaki et al., 1994). The stimulation of the three mediators with PMA from endometrial tissue collected throughout the menstrual cycle, and interactions between different stimulators of the chemokines and COX-2, was examined. Further, the effect of physiological concentrations of progesterone on the expression of IL-8, MCP-1 and COX-2 mRNA transcripts was investigated.



**Table 8**

Table summarising relevant *in vitro* induction and inhibition of chemokines

MEDIATOR	REGULATED BY	CELL TYPE	REFERENCE
<b>IL-8</b>	<b>Stimulators</b>		
	IL-1, TNF- $\alpha$	Endothelial cells	<i>Strieter et al, 1989</i>
	IL-1, TNF- $\alpha$ , LPS	Endothelial cells	<i>Brown et al, 1994</i>
	IL-1, TNF- $\alpha$ ,	Endometrial cells	<i>Arici et al, 1993</i>
	TGF- $\beta$ , serum	Endometrial cells	<i>Arici et al, 1996</i>
	<b>Inhibitors</b>		
	Progesterone, dexamethasone	Chorio-decidual and endometrial cells	<i>Kelly et al, 1994</i>
	Progesterone	Uterine-cervical cells	<i>Ito et al, 1994</i>
<b>MCP-1</b>	<b>Stimulators</b>		
	IL-1, TNF- $\alpha$ , LPS	Endothelial cells	<i>Brown et al, 1994</i>
	IFN- $\gamma$		
	PDGF	Fibroblasts	<i>Yoshimura et al, 1990</i>
	LIF, IL-6	Vascular smooth muscle cells	<i>Hartner et al, 1997</i>
	IL-1, TNF- $\alpha$ , IFN- $\gamma$	Endometrium	<i>Arici et al, 1995</i>
	hCG	Ovary	<i>Arici et al, 1997</i>
	<b>Inhibitors</b>		
	Oestradiol	Fibroblasts	<i>Kovacs et al, 1993, 1996</i>
	Progesterone	Chorio-decidual cells	<i>Kelly et al, 1997b</i>

**Table 9** summarising relevant *in vitro* induction and inhibition of COX-2

MEDIATOR	REGULATED BY	CELL TYPE	REFERENCE
<b>COX-2</b>	<b>Stimulators</b>		
	IL-1 $\beta$	Decidual cells	<i>Ishihara et al, 1992, 1995</i>
	IL-1, TNF- $\alpha$	Endometrium	<i>Chen et al, 1995b</i>
	Phorbol Ester, IL-1		<i>Cole et al, 1995</i>
	Serum	Endothelial cells	<i>Pritchard Jnr et al, 1994</i>
	RU486	Endometrium, decidua	<i>Kelly et al, 1986, Smith et al, 1987</i>
	<b>Inhibitory</b>		
	Progesterone	Decidual cells	<i>Ishihara et al, 1995</i>
	Dexamethasone	Endometrial cells	<i>Jacobs et al, 1994</i>

## 5.2 Materials and Methods

### Tissue Culture

Endometrial (n = 23) and decidual (n = 12) biopsies were transported from the operating theatre in RPMI culture medium on ice. The tissue was roughly chopped with a sterile scalpel blade into fragments of 1-2 mm<sup>2</sup>. The pieces of endometrium were distributed equally between 4 wells of a 24 well culture plate, each containing complete medium (RPMI supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, gentamycin). The tissue was incubated for 2 hours at 37°C, CO<sub>2</sub> and the complete medium was supplemented with the following treatments:

1. Control - no additional treatment
2. Phorbol 12-Myristate 13-Acetate (PMA 0.5µM) in complete medium
3. Progesterone (0.25µM) in complete medium
4. Both Phorbol 12-Myristate 13-Acetate (PMA 0.5µM) + Progesterone (0.25µM)

A further subset of endometrial biopsies (n = 8) were collected and divided into four treatment groups. One portion was snap frozen at time of collection, and the remaining three were cultured for 2 hours under similar conditions as before with the following treatments:

1. Unsupplemented RPMI medium (no FCS)
2. FCS (10%) supplemented RPMI (complete medium)
3. Complete medium supplemented with PMA (0.5µM) and FCS (10%)

After the culture period, the endometrium and decidua samples were placed into sterile eppendorf tubes with 1 ml of Ultraspec™ RNA extraction medium and total RNA was extracted as described previously (Chapter 2, section 2.24).

**Table 10**

Table illustrating the numbers of endometrial and decidual biopsies utilised in the present study, and the stage of the cycle at the time of biopsy.

STAGE OF CYCLE	DAYS FROM LMP	NUMBER OF BIOPSIES
Menstrual	d1-4	4
Proliferative	d5-13	5
Ovulatory / Early Secretory	d14-18	5
Mid Secretory	d19-24	5
Late Secretory	d25-mens	4
First Trimester Decidua	8-10 weeks amenorrhoea	12

**Semi-quantitative RT-PCR**

RT-PCRs for GAP-DH, IL-8, MCP-1 and COX-2 were conducted on RNA extracted from non-pregnant and pregnant endometrium cultured in the above conditions. RNA loading was standardised to 60ng following repeated GAP-DH RT-PCR and ELISA (sections 2.25-2.26). The same RNA stocks were then reverse transcribed and cDNA was subjected to PCR for IL-8, MCP-1 and COX-2. A quality control RNA sample (QC) known to contain mRNA for the inflammatory mediators was included in each RT-PCR. Quantitative ELISAs were conducted to measure the relative amount of final amplicon for each mediator, and these were expressed as the ratio of sample signal : control signal.

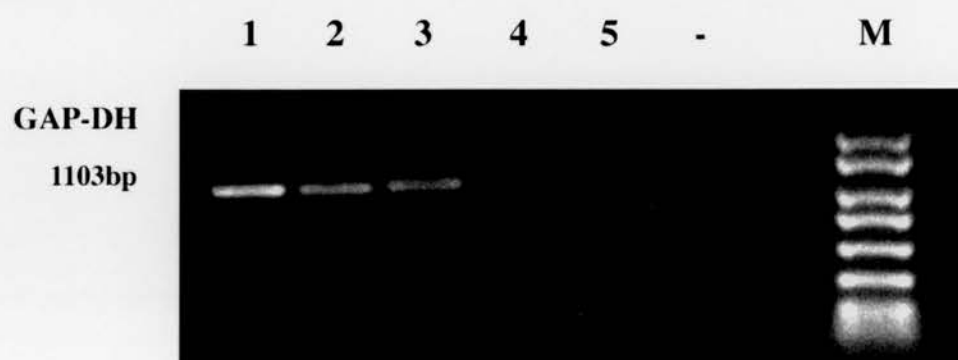
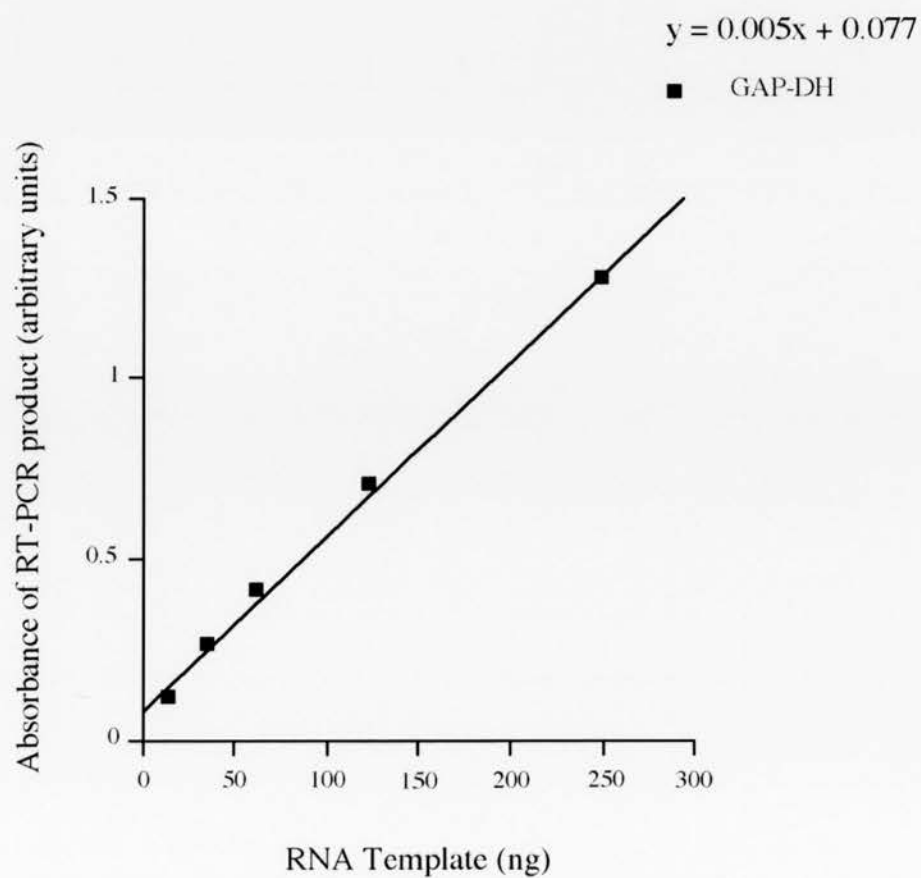
### 5.3 Results

RNA template loading into the mediator RT-PCRs was standardised by measurement of GAP-DH expression levels. Bands of equal intensity were achieved following GAP-DH RT-PCR and gel electrophoresis (figure 41), and readings of 60ng (+/- 10ng) were generated by ELISA.

IL-8, MCP-1 and COX-2 RT-PCRs produced single bands of the correct molecular weight (IL-8 298bp, MCP-1 210bp, COX-2 350bp). Large differences in mediator expression levels were observed between uncultured samples and endometrium cultured for 2 hours at 37°C. Different PCR cycling protocols were therefore required to obtain products during the exponential phase of amplification, e.g. for IL-8 RT-PCR, 23 PCR cycles were required for uncultured samples compared with 32 cycles for cultured samples (table 3). Limited comparison of expression levels could therefore be conducted between the two groups of samples. A comparison of IL-8, MCP-1 and COX-2 mRNA levels however, between a representative uncultured and cultured endometrial sample is shown in Figures 1, 2 and 3, utilising PCR protocols optimised for cultured samples.

**Figure 41** Standard curve for GAP-DH RT-PCR and ELISA, included with cultured endometrial samples. Loading of mRNA into RT-PCR reaction is shown along the x axis, and absorbance of RT-PCR product along the y axis. A linear relationship is achieved, producing the equation  $y = 0.005x + 0.077$ , enabling RNA loading of samples to be calculated.



**A****B**

## **1 Effect of culture on endometrial mediator expression**

The expression levels of all three mediators in uncultured endometrium varied according to the stage of the menstrual cycle (figures 42, 44, 45 and 46), in agreement with previous findings (Chapter 3 section 3.3). An increase in IL-8 mRNA levels was detected following culture in all samples examined, although again with much variability between endometrium collected from different stages of the menstrual cycle. In particular, the magnitude of elevation following culture with 10% FCS was considerably less in the secretory phase samples analysed, compared with menstrual and proliferative samples. COX-2 expression levels followed a matching pattern, with only a slight increase in mRNA levels after stimulation in secretory phase biopsies, compared with proliferative biopsies. Similarly, maximal MCP-1 stimulation by culture was observed in mid proliferative endometrium.

**Figure 42** Effect of culture on IL-8 and MCP-1 mRNA expression. This gel composite illustrates the marked effect culture has on the expression of mRNA for the chemokines. Tissue samples were divided into two at the time of collection, so that RNA could be extracted from one immediately whilst the remaining portion was cultured for 2 hours in serum supplemented culture medium prior to RNA extraction. The RT-PCR product from the cultured and uncultured samples are presented in neighbouring wells. A dramatic upregulation of IL-8 mRNA expression is detected after the 2 hour culture period. A similar pattern is observed for MCP-1, although not so consistent. For both chemokines:

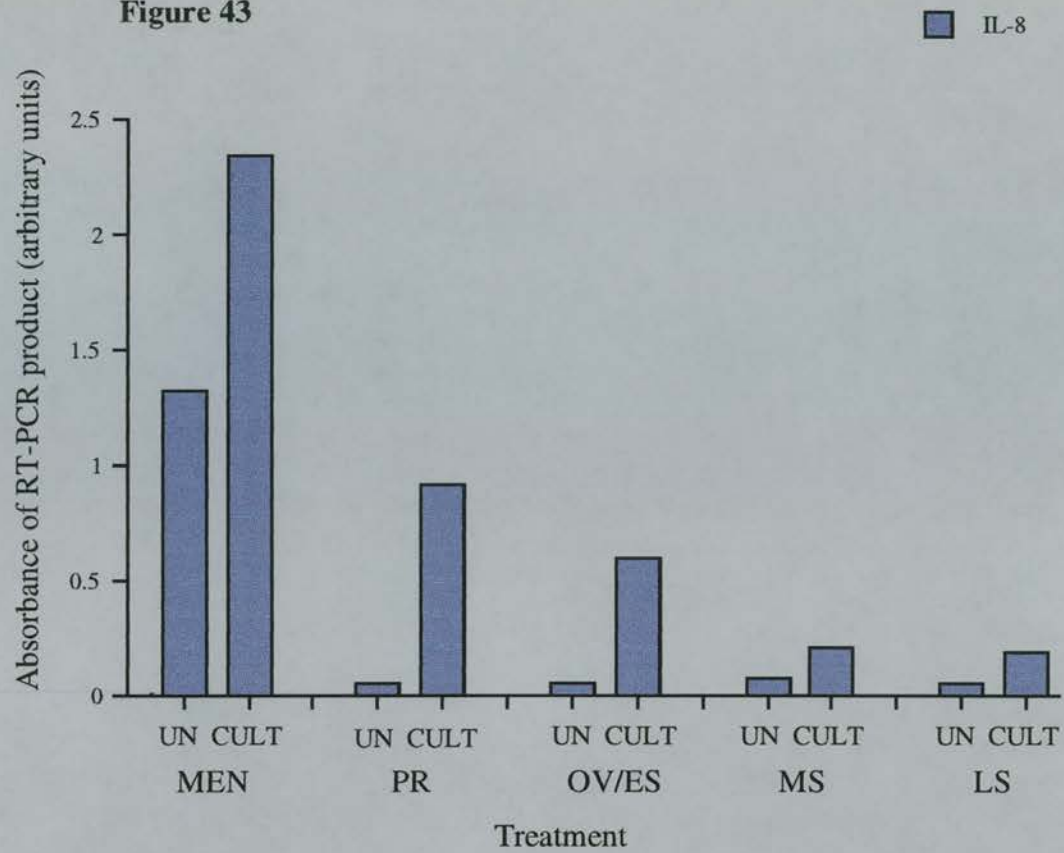
- 1 = early proliferative phase endometrium cultured
- 2 = early proliferative phase endometrium uncultured
  
- 3 = menstrual phase endometrium cultured
- 4 = menstrual phase endometrium uncultured
  
- 5 = late proliferative phase endometrium cultured
- 6 = late proliferative phase endometrium uncultured
  
- 7 = mid secretory phase endometrium cultured
- 8 = mid secretory phase endometrium uncultured



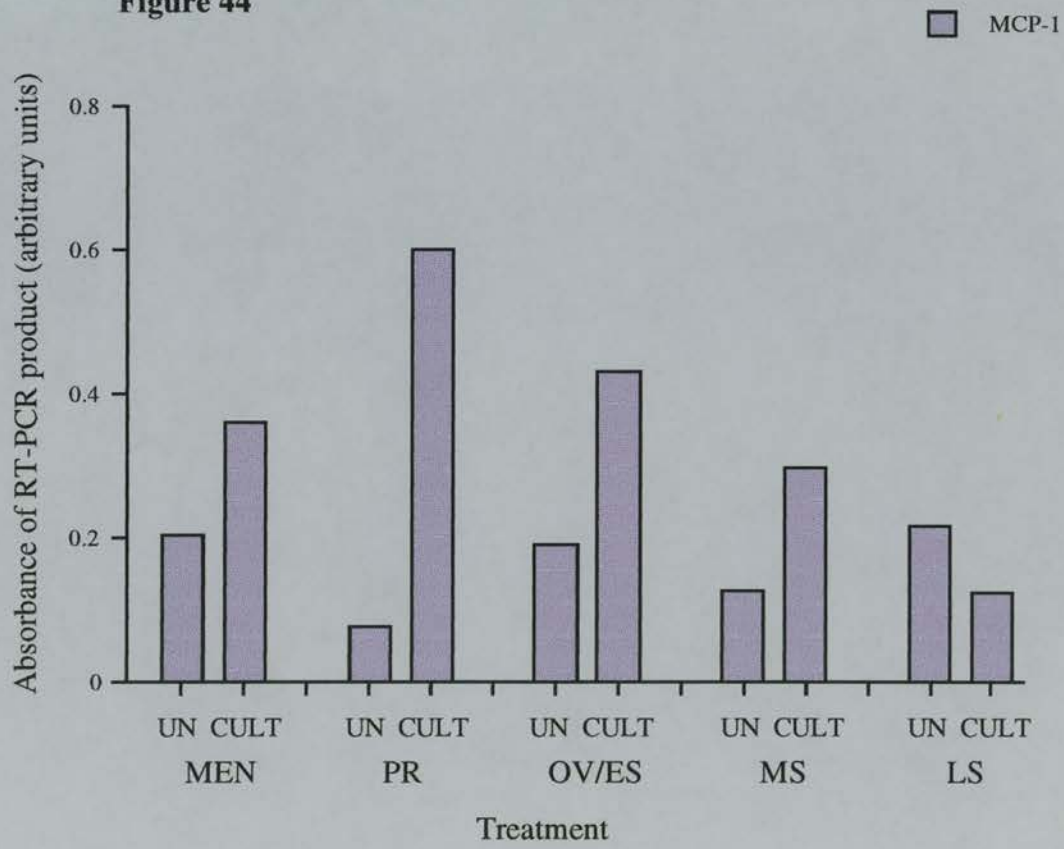
**Figure 43** Effect of culture on IL-8 mRNA expression levels. This histogram demonstrates the increase in IL-8 mRNA expression levels after a 2 hour culture period (CULT), compared to uncultured (UN) tissue. Marked differences were visible in the degree of stimulation following culture in endometrium collected from different stages of the menstrual cycle (MEN = menstrual, PR = proliferative, OV/ES = ovulatory / early secretory, MS = mid secretory, LS = late secretory). Most noticeable was the reduced ability for upregulation of IL-8 mRNA expression by culture in secretory endometrium.

**Figure 44** Effect of culture on MCP-1 mRNA expression levels. Slight variations were detected in the expression levels of MCP-1 mRNA in uncultured (UN) endometrium collected at different stages of the cycle (MEN = menstrual, PR = proliferative, OV/ES = ovulatory / early secretory, MS = mid secretory, LS = late secretory). After culture in the presence of serum (CULT), a stimulation in MCP-1 mRNA levels was apparent in all but the late secretory phase endometrium. As for IL-8, the degree of stimulation was greatest in proliferative phase endometrium.

**Figure 43**



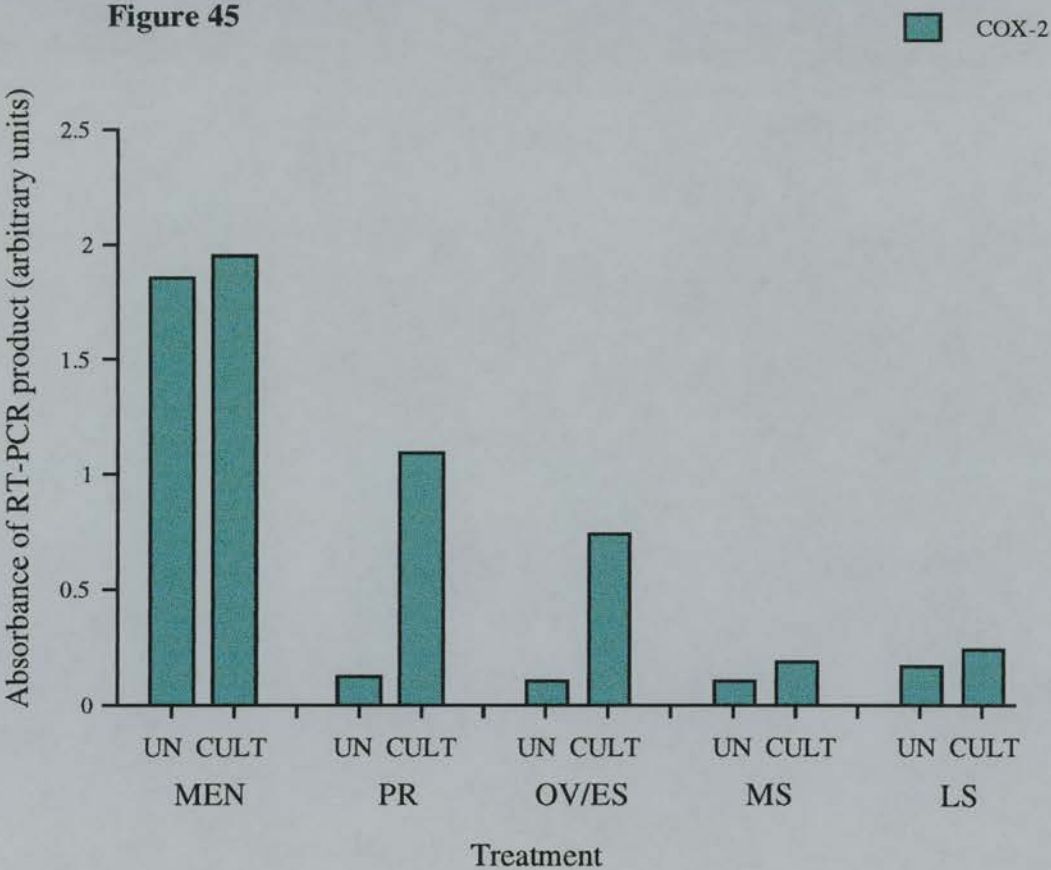
**Figure 44**



**Figure 45** Effect of culture on COX-2 mRNA expression levels. Elevated numbers of COX-2 mRNA transcripts were detected in uncultured (UN) menstrual phase endometrium (MEN), compared to those at all other stages examined. Following culture (CULT) with 10% serum, a marked elevation in COX-2 mRNA was observed in proliferative (PR) and ovulatory / early secretory phase (OV/ES) endometrium. Similar treatment of menstrual and mid-late secretory phase (MS and LS) endometrium had very little effect.



**Figure 45**



## **2. Effect of serum on endometrial mediator expression**

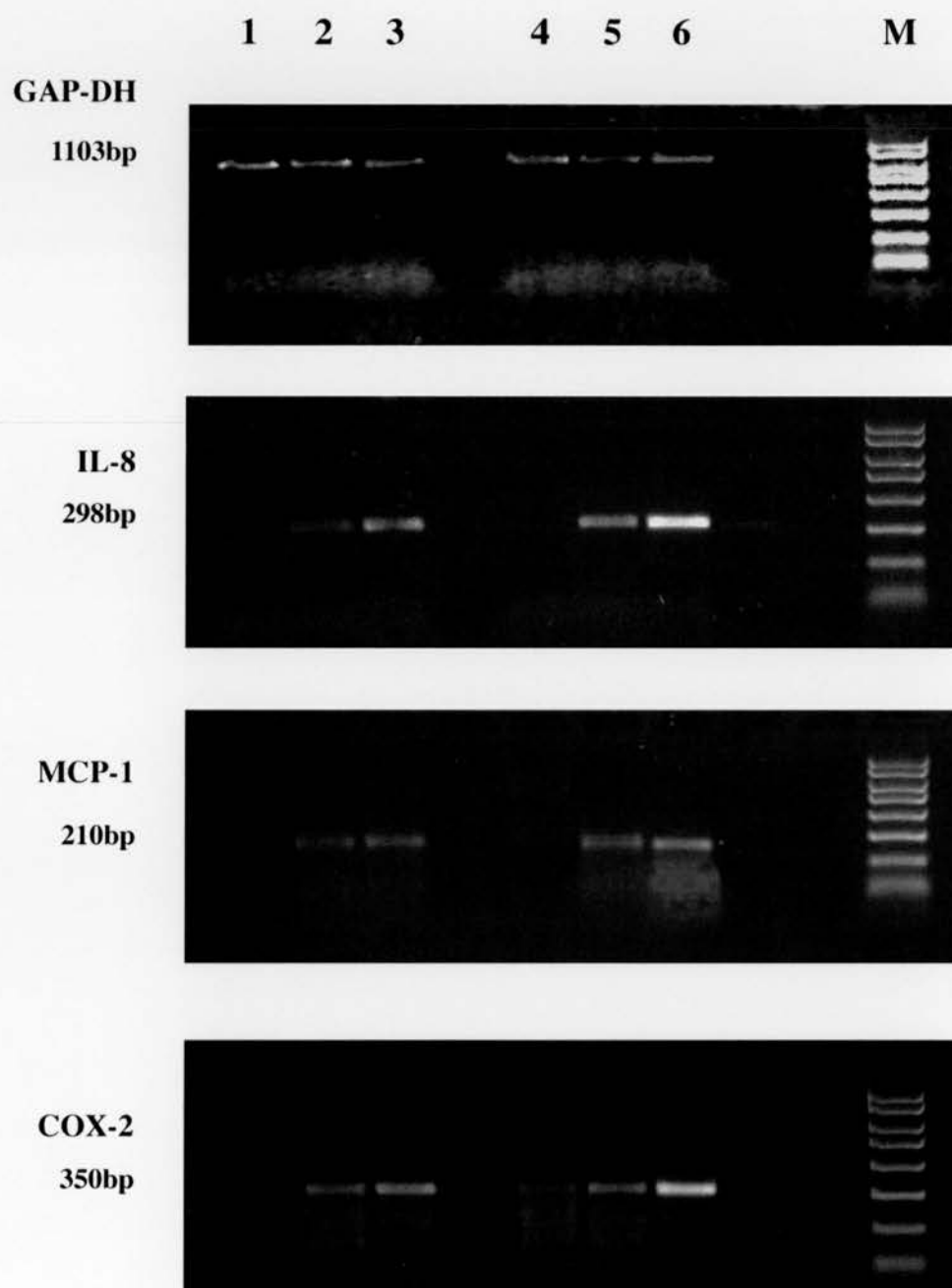
To investigate if the elevation in mediator mRNA levels observed was due to the presence of 10% FCS in the culture medium, a series of experiments were conducted involving culturing endometrium in the presence and absence of FCS (figure 46). Considerable variations were detected between the samples, producing confusing results when presented as average results. Representative results are therefore displayed in figures. In both mid proliferative and mid secretory endometrium, IL-8 mRNA expression levels increased when cultured in unsupplemented RPMI medium, compared to expression levels in uncultured biopsies (figure 47). A further increase was demonstrated with the addition of 10% FCS to the culture medium. A similar augmentation of MCP-1 and COX-2 expression levels with FCS was detected in mid proliferative endometrium, although in secretory phase endometrium, the pattern was not so clear.

## **3. Induction of mediator mRNA by PMA**

The effects on mediator expression of PMA supplementation to the culture medium was investigated (figure 46). Figure 48 shows the results from three representative endometrial biopsies. With the exception of the menstrual endometrial sample, previously demonstrated to have high levels of IL-8 and COX-2 mRNA transcripts, mediator expression in uncultured endometrium (mid proliferative and mid-secretory phases) was practically undetectable using this protocol. In general, the elevation in IL-8 and MCP-1 mRNA expression was augmented by the presence of PMA and serum in the culture medium. A further increase in the amount of COX-2 mRNA was not detected following treatment with PMA when compared to with serum alone.

**Figure 46** Induction of inflammatory mediator mRNA expression *in vitro*. This composite illustrates the induction of IL-8, MCP-1 and COX-2 mRNA from proliferative phase endometrium. The upper panel (GAP-DH expression) illustrates the equal loading of RNA samples. RNA extracted from (1) uncultured tissue, is compared with (2) tissue cultured for two hours in unsupplemented medium and (3) tissue cultured in culture medium supplemented with 10% FCS. mRNA for all three mediators is stimulated in the absence of serum, with a further elevation in the number of transcripts when serum is present.

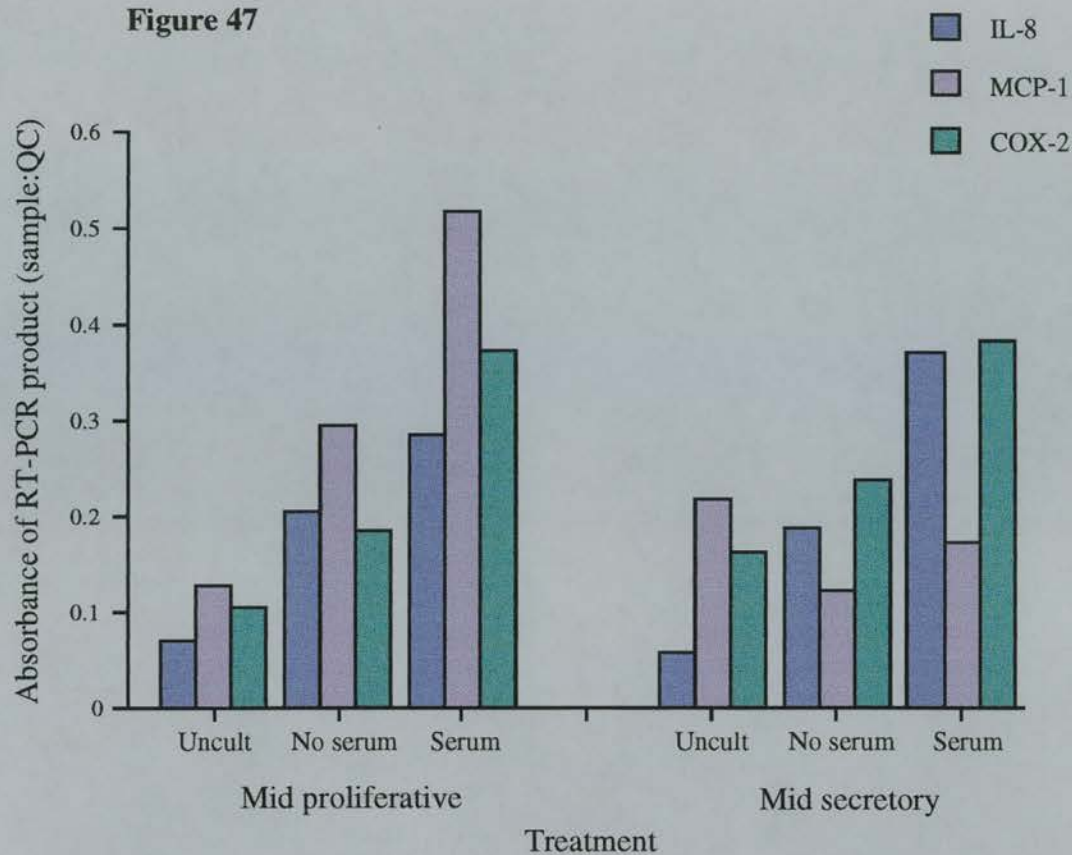
Uncultured endometrium inflammatory mediator mRNA content (4) is also compared with that cultured with 10% FCS (5) and with 10% FCS plus PMA (6). Again, IL-8, MCP-1 and COX-2 are induced with exposure to serum, and a further increase is evident with addition of PMA to the culture medium.



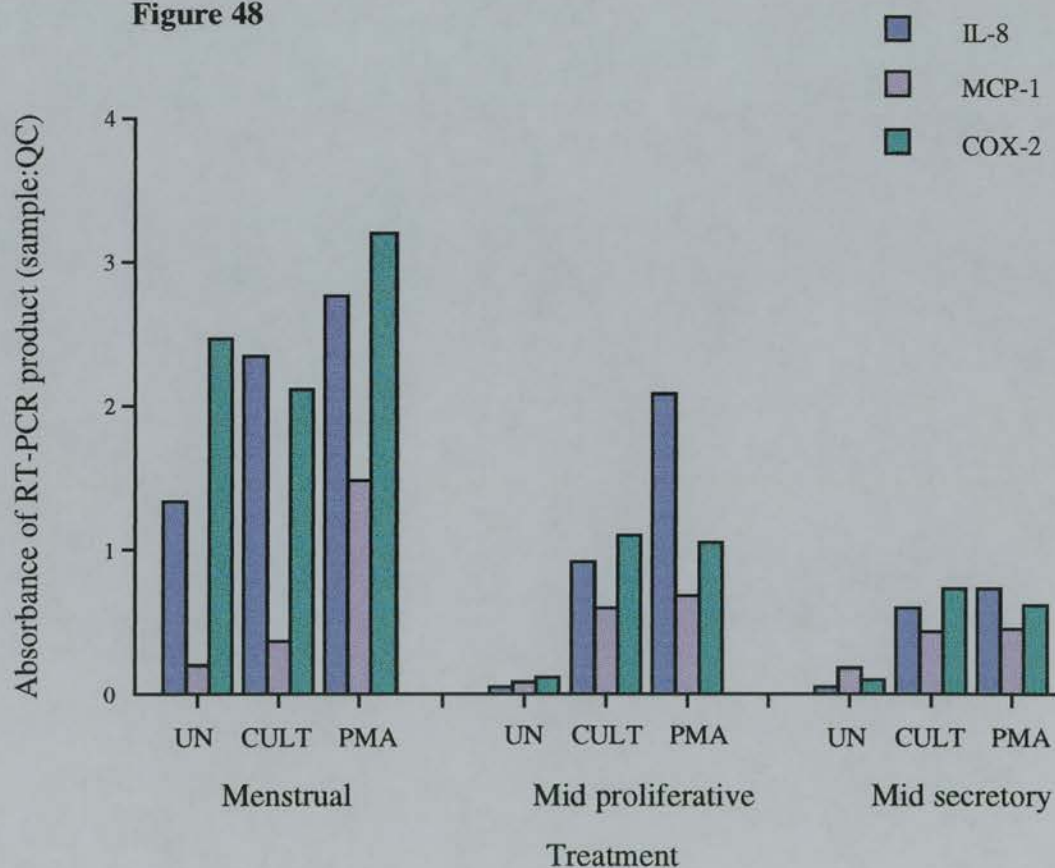
**Figure 47** Effect of culture in the presence and absence of serum on inflammatory mediator mRNA expression levels. In mid proliferative phase endometrium, IL-8, MCP-1 and COX-2 mRNA expression was stimulated by culturing without serum. A further stimulation was apparent when culture medium was supplemented with 10% FCS. A similar pattern was observed for IL-8 and COX-2 in mid secretory phase endometrium.

**Figure 48** Effect of culture in the presence of serum, with or without PMA, on mediator mRNA expression. At all stages of the menstrual cycle, a clear stimulation of IL-8 mRNA expression by serum (CULT) is observed compared to uncultured tissue (UN), which is augmented by the addition of PMA. MCP-1 is similarly induced in response to culture, although a synergy between serum and PMA is less clear in the proliferative and secretory endometrium. mRNA for COX-2 is increased following culture, with no further stimulation by PMA observed.

**Figure 47**



**Figure 48**



#### **4. Effect of progesterone on endometrial mediator expression**

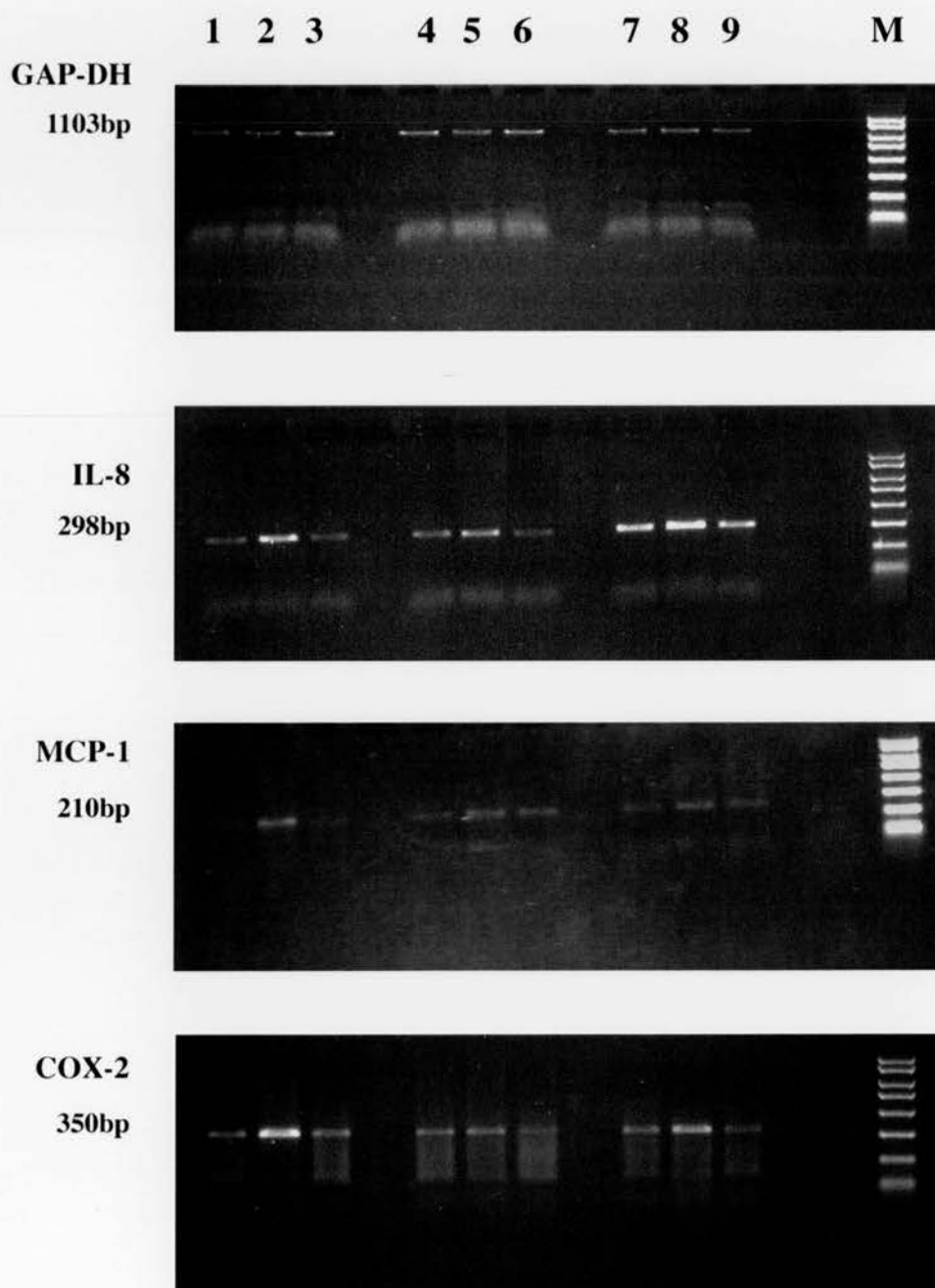
Exposure of endometrium to progesterone ( $5 \times 10^{-7}\text{M}$ ) during a 2 hour culture period had limited effect on the expression levels of IL-8, MCP-1 and COX-2 (figures 49, 50, 51 and 52). The numbers of mRNA transcripts detectable by RT-PCR were comparable with those without progesterone (control). In some cases, particularly in the late secretory phases, slight decreases in IL-8, MCP-1 and COX-2 mRNA levels were detected, but these changes were not significant. The addition of progesterone to tissue cultured with PMA caused no alterations in the mediator expression levels (data not shown).

#### **5. Effect of progesterone on decidual mediator expression**

Similarly, no significant changes in IL-8, MCP-1 or COX-2 mRNA expression were observed in decidua cultured with progesterone. The results from representative samples are illustrated in figures 54, 55, 56 and 57, showing an elevation in decidual IL-8, MCP-1 and COX-2 expression in response to PMA, but no significant decrease in the presence of exogenous progesterone. Decidual expression chemokines and COX-2 exposed to both PMA and progesterone were comparable to with PMA alone (data not shown).



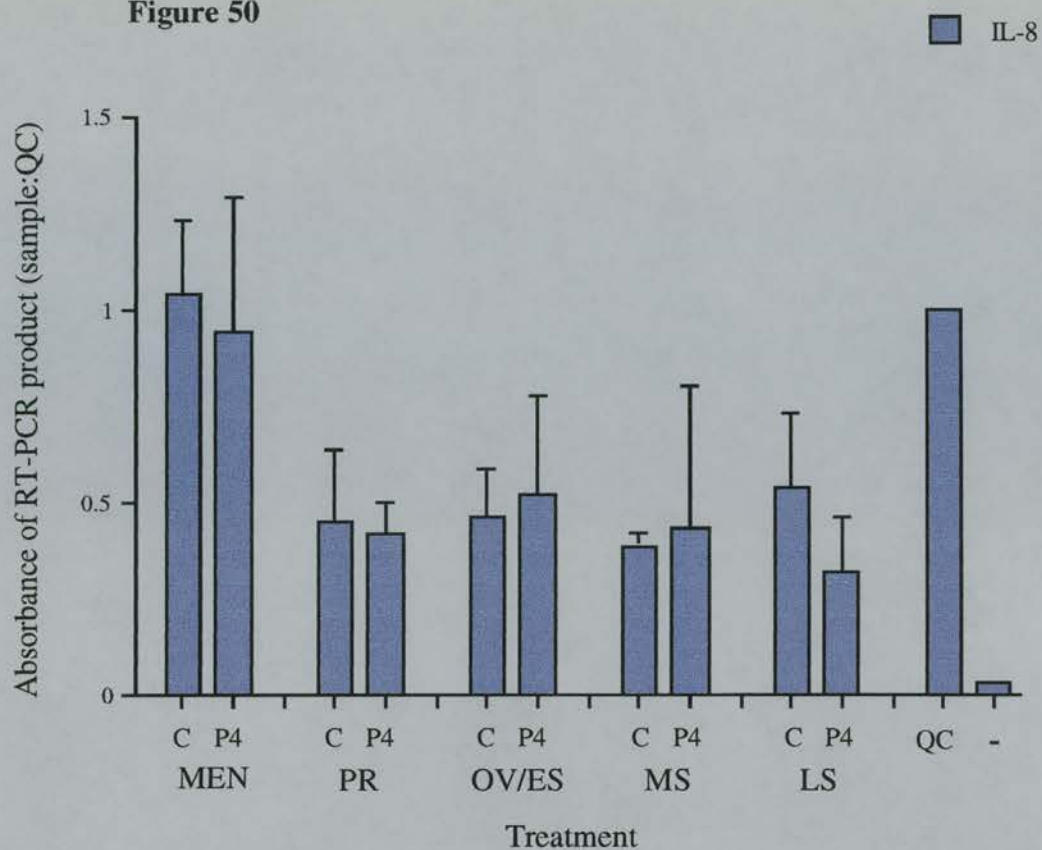
**Figure 49** Effect of progesterone on IL-8, MCP-1 and COX-2 mRNA expression *in vitro*. Three representative endometrial samples are shown in the composite, collected from the proliferative, secretory and menstrual phase (left - right). For each sample, tissue cultured with 10% FCS (1, 4, 7) is compared with tissue cultured with 10% FCS plus PMA (2, 5, 8), and tissue cultured with 10% FCS and progesterone (3, 6, 9). As described earlier, a stimulation of mediator mRNA is generally observed with the addition of PMA, but progesterone supplementation had no consistent effect on IL-8, MCP-1 or COX-2 mRNA expression.



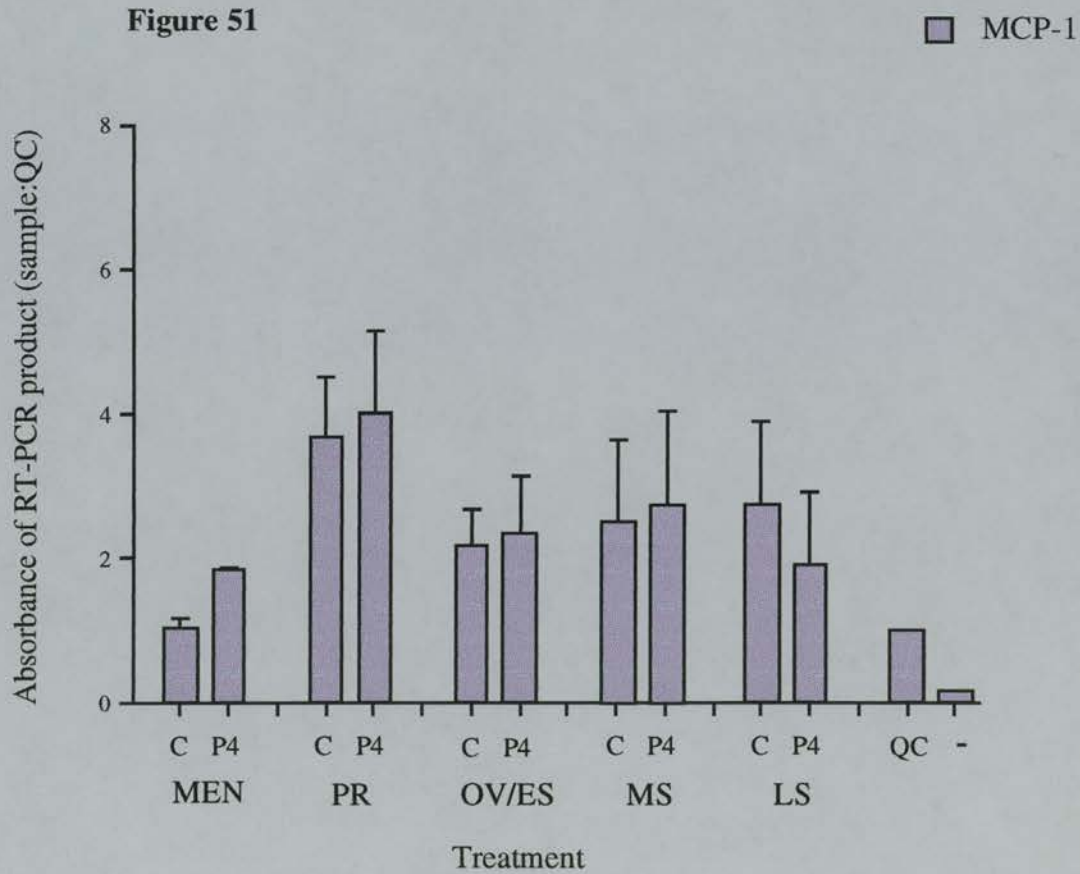
**Figure 50** Effect of progesterone on IL-8 mRNA expression. Marked variations were observed in IL-8 expression at different stages of the menstrual cycle, and patient to patient variations were also noted (Y axis error bars represent SEM). Addition of progesterone to the culture medium had little effect on mRNA levels.

**Figure 51** Effect of progesterone on MCP-1 mRNA expression. As for IL-8 mRNA expression levels, no significant differences in the numbers of MCP-1 mRNA transcripts were detected following culture with progesterone.

**Figure 50**

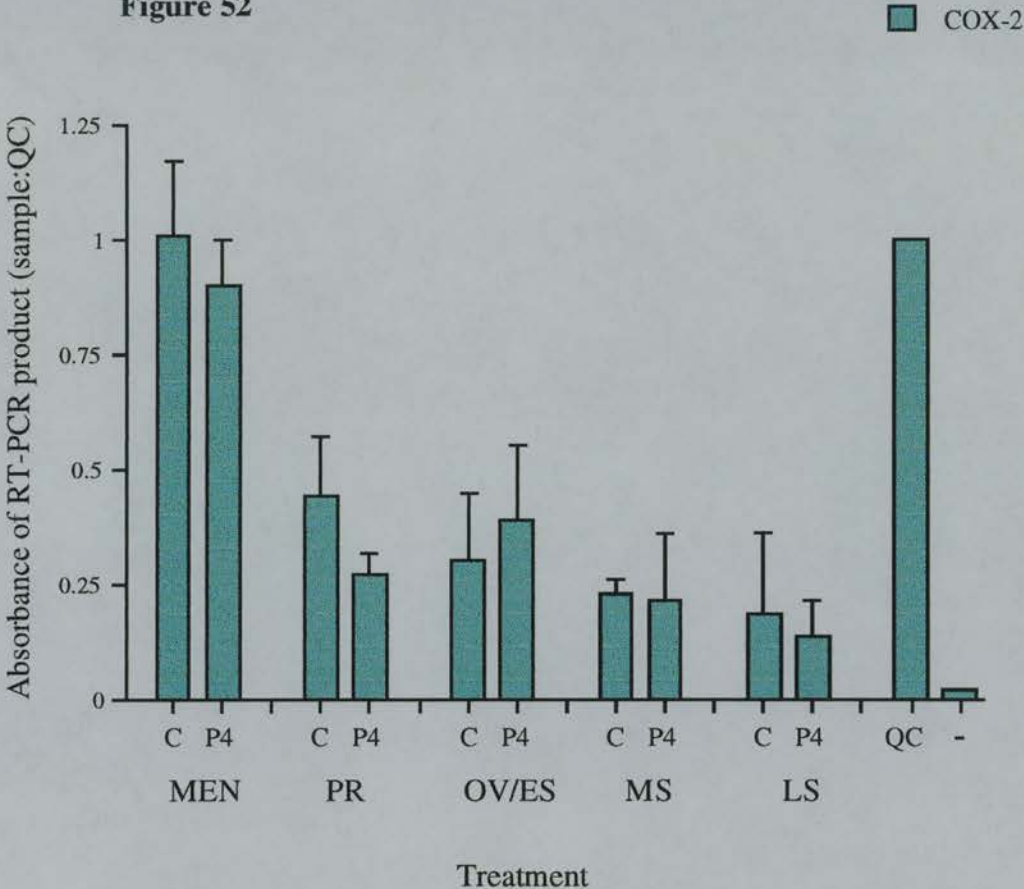


**Figure 51**



**Figure 52** Effect of progesterone on COX-2 mRNA expression. Menstrual phase endometrium possessed high numbers of COX-2 mRNA transcripts, compared to samples collected throughout the menstrual cycle. No significant changes in COX-2 expression were identified with exposure to progesterone for 2 hours.

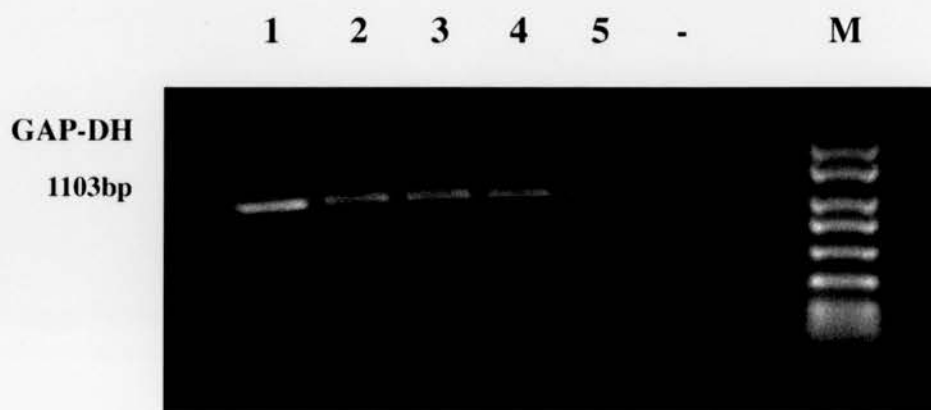
Figure 52



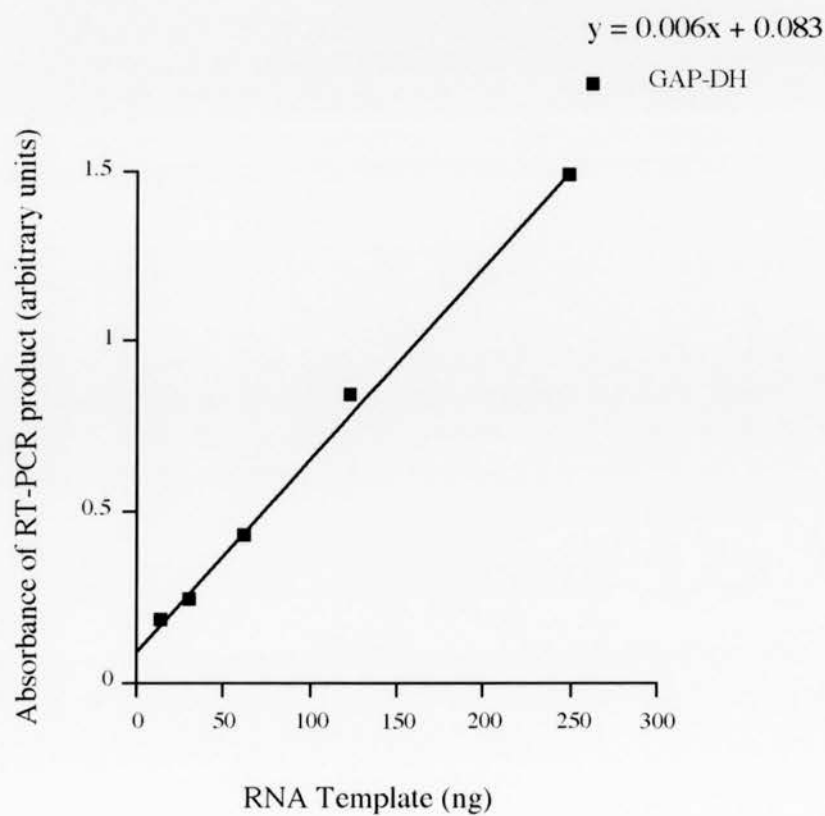
**Figure 53** Standard curve for GAP-DH RT-PCR and ELISA, included with cultured decidua RNA samples. Standards ranging between 250ng and 17.5ng were loaded into the RT-PCR, producing the standard curve after measurement of product yield by ELISA. This generated the equation  $y = 0.006x + 0.038$ , which was thereafter used to correct the amount of RNA loading into the RT-PCR.



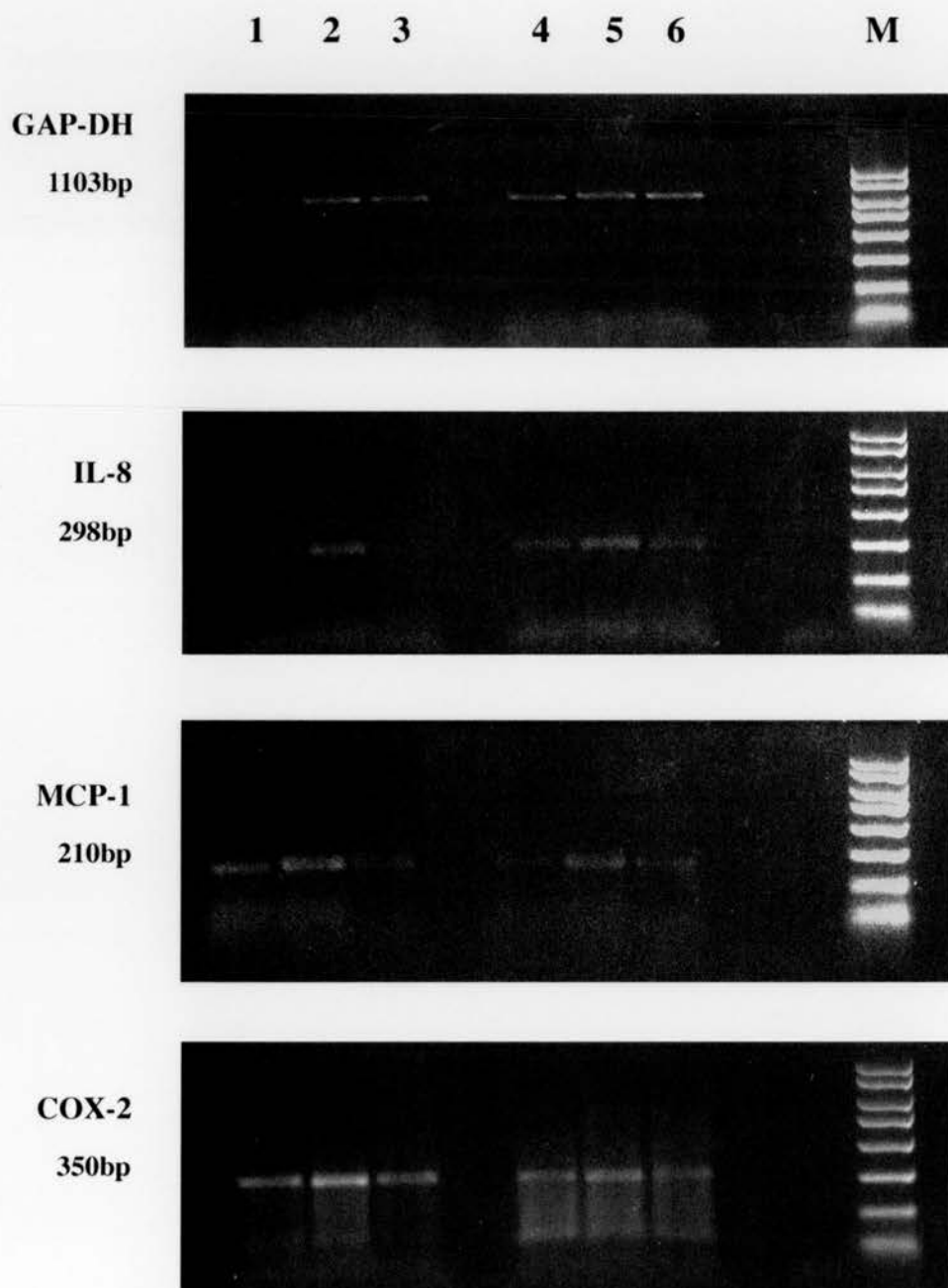
**A**



**B**



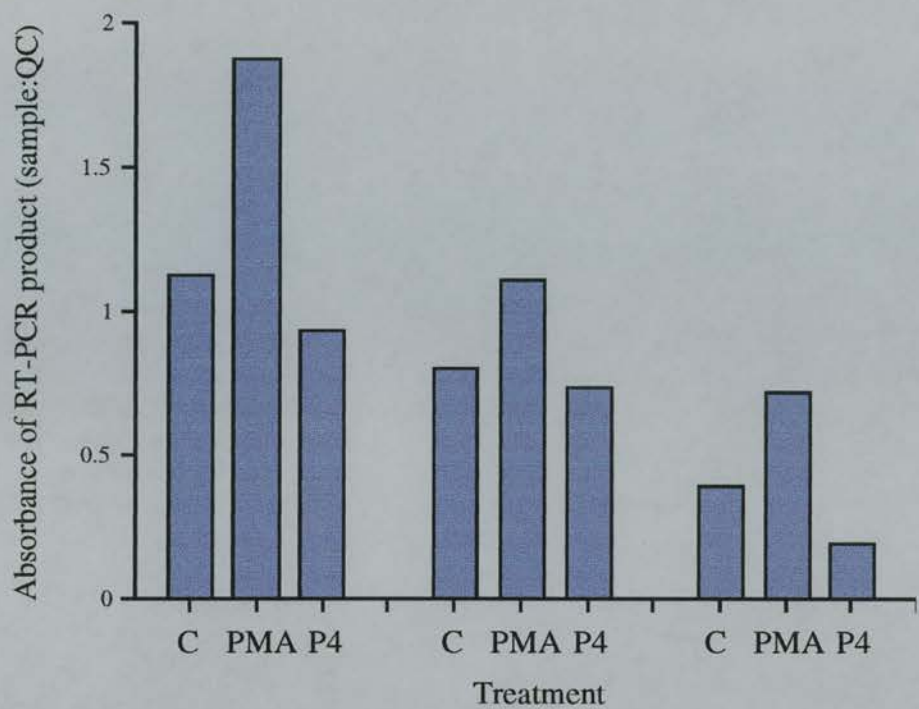
**Figure 54** Effect of progesterone on decidual inflammatory mediator expression. Two representative decidual samples amplified for GAP-DH, IL-8, MCP-1 and COX-2 are presented. Low numbers of messenger transcripts were detected when decidua was cultured just in the presence of 10% FCS (1 & 4). Addition of PMA to the culture medium stimulated mRNA expression for the chemokines and COX-2 (2 & 5). Addition of progesterone to the culture medium had little effect on the degree of expression of IL-8, MCP-1 or COX-2.



**Figures 55 and 56** Effect of culture with PMA and progesterone on decidual IL-8 and MCP-1 mRNA expression. Marked variations were apparent between individual decidual samples, and thus representative samples are shown in this histogram. Treatment with PMA stimulated IL-8 mRNA expression levels. Progesterone had no significant effect on expression levels.

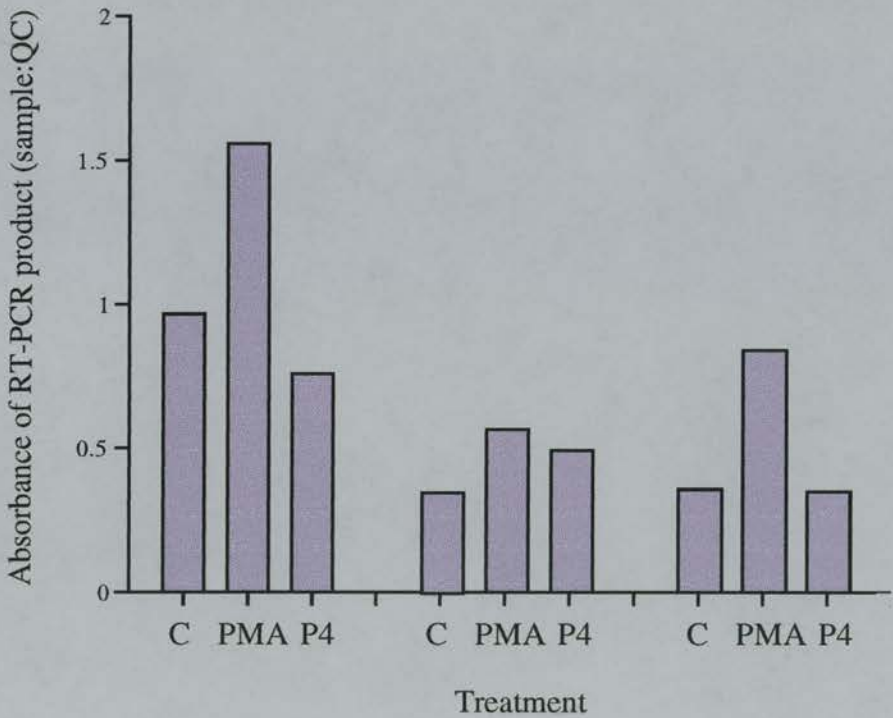
**Figure 55**

■ IL-8



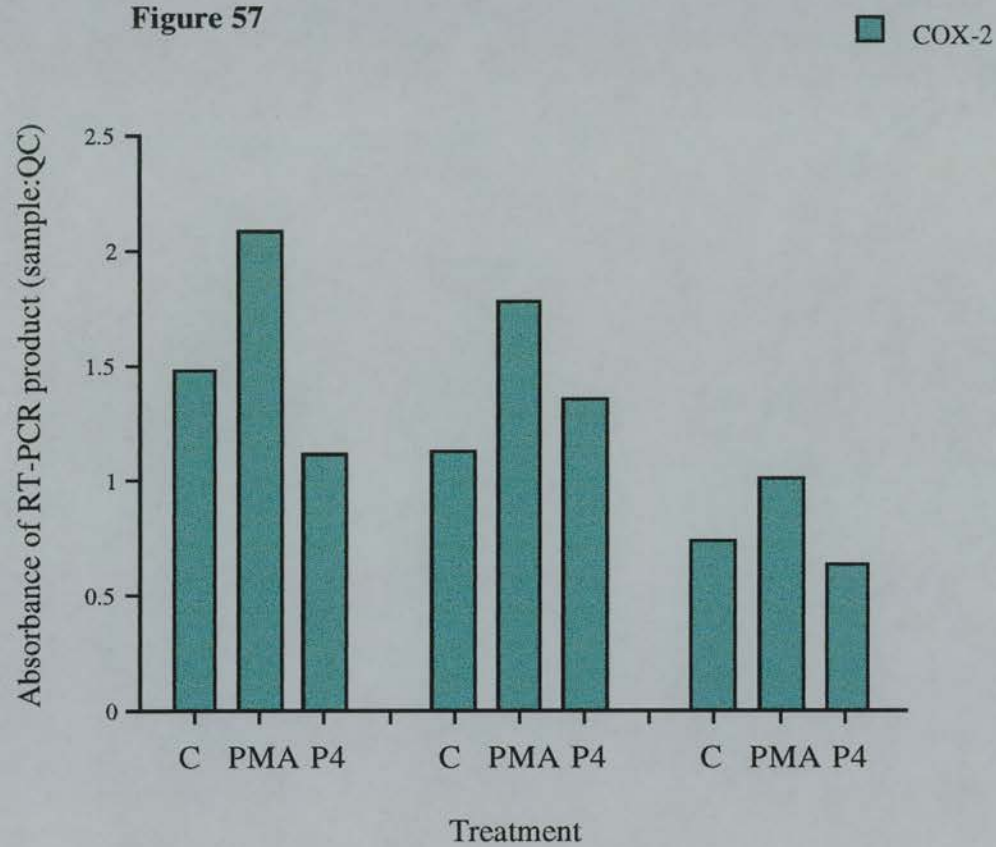
**Figure 56**

■ MCP-1



**Figure 57** Effect of culture with PMA and progesterone on decidual COX-2 mRNA expression. Again, distinct variations were observed between individual samples. An increase in COX-2 mRNA levels was apparent after stimulation with PMA, but no changes were detectable following treatment with progesterone.

**Figure 57**





## 5.4 Discussion

A model of *in vitro* culture of endometrium and decidua was employed to investigate and examine the mRNA expression of the inflammatory mediators. In this study, IL-8, MCP-1 and COX-2 mRNA expression levels were examined following a 2 hour culture period in the presence or absence of PMA and or progesterone. Induction of all three mediators was recognised even in the absence of PMA, and this was presumed to be due to the presence of 10% FCS in the culture medium. However, a representative sample of tissues were cultured in unsupplemented RMPI and a substantial stimulation of mediator message was detected. This could be explained by the trauma of tissue collection and subsequent mechanical damage received whilst tissue was cut into pieces. A similar stimulation of COX-2 mRNA was reported due to damage received by vascular smooth muscle cells following balloon de-endothelialisation (Pritchard Jnr et al., 1994). Within 2 hours, a marked increase in COX-2 mRNA was detectable, declining to basal levels after 6 hours.

The addition of FCS to the culture medium produced an elevation in mediator mRNA expression, above that detected in its absence and a further stimulation of IL-8 was obtained when the medium was supplemented with PMA. This suggests a synergistic action between PMA and FCS in the stimulation of IL-8 mRNA. An elevation in IL-8, MCP-1 and COX-2 mRNA was detected in all tissues examined following culture with or without stimulants, although the extent did vary considerably with the stage of the menstrual cycle. Notably, the degree of stimulation was markedly reduced for all mediators in secretory phase compared to proliferative endometrium. This would suggest inherent differences in the regulation of chemokines and COX-2 in proliferative and secretory phases. A logical explanation is that the expression of mediators in the secretory phase is suppressed by endogenous progesterone, which dominates during the second half of the menstrual cycle. This effect appears to persist during and after the 2 hour culture period.

No attempts were made in this study to determine the mechanism behind the elevated numbers of mRNA transcripts following culture. There is an inconsistency in the literature regarding whether the stimulation consistently reported is due to increased transcription of the mediators or to stabilisation of existing mRNA transcripts. Arici et al. describe a system whereby an increase in *de novo* IL-8 mRNA synthesis is not the major contributory factor, but instead stabilisation of pre-

existing transcripts occurs (Arici et al., 1993). An opposing explanation was offered by Dudley (1993) who demonstrated de novo transcription of decidual IL-8 after IL-1 $\alpha$  treatment (Dudley et al., 1993). Further inconsistencies appear in the literature as to the mechanism of stimulation of MCP-1 (Sica et al., 1990) and COX-2 (Jacobs et al., 1994; Ristimaki et al., 1994).

IL-8, MCP-1 and COX-2 are all believed to be immediate-early response genes (Maier et al., 1990; Oppenheim et al., 1991; Freter et al., 1995), which are rapidly transcribed in response to stimuli. To allow greater sensitivity of the response, the mRNA transcripts have very short half lives due to the presence of an AUUUA motif in the 3' untranslated region, which confers rapid degradation (Caput et al., 1986; Shaw et al., 1986; Matsushima et al., 1988). Several substances, including IL-1, TNF- $\alpha$  and serum, have been reported to extend the half lives of the mRNA by stabilisation (Malter, 1989; Stoeckle, 1991). Either mechanism could account for the elevated levels observed in the present study. It is interesting that the tissues retain a "memory" for the endogenous steroid environment prevailing prior to biopsy, in that mediator levels vary according to the stage of the menstrual cycle. Moreover, the degree of stimulation following culture with or without serum and / or PMA differs in proliferative and secretory endometrium. One explanation would be that stimulation of the chemokines and COX-2 by culturing causes stabilisation of existing transcripts. It is possible, however, that the capacity to synthesise the inflammatory mediators is reduced even after removal from a progesterone dominated environment.

No changes in chemokine or COX-2 expression levels were detected after treatment with progesterone. A number of explanations can be offered to understand this unexpected finding.

1. Most research into the downregulation of chemokines or COX-2 by progesterone have measured the production of protein rather than mRNA. The possibility therefore exists that a post-transcriptional mechanism is responsible for the inhibition. Translational or post-translational mechanisms could be operating to reduce the levels of immunoreactive protein. This would, however, contradict the findings described in the previous chapter whereby an increase in mRNA levels for IL-8 and COX-2 were identified 48 hours after progesterone withdrawal in an *in vivo* model. Furthermore, parallel investigations into the downregulation of COX-2 by dexamethasone have concluded that reduced transcription or destabilisation of mRNA is responsible.

2. An indirect regulation may be occurring, not involving direct interaction of progesterone with promoter regions of the gene. This has previously been described in decidual cells, where treatment with cyclohexamide, an inhibitor of protein synthesis, caused an elevation in IL-8 mRNA expression (Dudley et al., 1993). The same was reported for COX-2, where addition of actinomycin D stimulated PGF<sub>2α</sub> (Smith et al., 1988). This indicates the requirement for intermediate proteins which would mediate the inhibition and interact with regulatory elements. All three mediators do however possess putative progesterone / glucocorticoid response elements on the promoter sequences (Matsushima et al., 1989; Mukaida et al., 1989; O'Banion et al., 1992), thus making a direct regulation possible. Additionally, the cell types of interest, particularly the perivascular cells, all express PgR (Perrot-Applanat et al., 1994). The validity of this argument is reduced by the experimental design utilised, as whole tissue was treated. This model is advantageous in certain respects as the tissue has not been subdivided into cellular components and therefore any paracrine interactions from neighbouring cell types should still be allowed. If certain cell types were isolated, a disruption in physiological regulation of three mediators would be more likely.

3. It is possible that an inhibition or down regulation of IL-8, MCP-1 and COX-2 by progesterone cannot be detected in a 2 hour culture period. Although the mediators are clearly induced in this length of time, it is possible that the mechanisms involved in their downregulation are slower. Alternatively, because digested cells were not utilised, the time required for permeation of substances into the tissue pieces may be longer than originally thought. Several publications have highlighted the transient nature of chemokine and COX-2 induction (Strieter et al., 1989; Arici et al., 1993; Pritchard Jnr et al., 1994; Arici et al., 1995; Hartner et al., 1997). IL-8 mRNA levels in cultured endometrial cells decrease spontaneously during the culture period. Arici et al describe a reduction in IL-8 mRNA levels from as early as 3-6 hours of culture (Arici et al., 1993), and comparable data in the literature support a similar degradation of MCP-1 (Sach et al., 1997) and COX-2 mRNA (Pritchard Jnr et al., 1994). It is important to note that the aim of this study was to isolate the primary response of the treatments on mediator expression. It was therefore important in this study to avoid complication between the natural degradation of mediator mRNA and the possible inhibition mediated via progesterone.

4. As discussed above, the mechanism behind the elevated levels mRNA in tissue cultured with serum or PMA is unknown, with previous reports of both

stimulation of *de novo* transcription and stabilisation of existing transcripts synthesised prior to the culture period. It is likely however, that an inhibition of the chemokines and COX-2 by glucocorticoids and progesterone would be mediated through a suppression of transcription. Recent evidence implicates the repression of Nuclear Factor- $\kappa$ B (NF- $\kappa$ B), which is a transcriptional activator of many inflammatory genes including IL-8, COX-2, IL-6 (van der Burg et al., 1997). Following stimulation by proinflammatory mediators, such as IL-1, TNF- $\alpha$  and phorbol esters, cytoplasmic NF- $\kappa$ B disassociates from inhibitory molecule I $\kappa$ B, and is translocated to the nucleus. NF- $\kappa$ B then binds to target sequences in the promoter regions of responsive genes and activates transcription. Glucocorticoids and progesterone may repress NF- $\kappa$ B activation of transcription, either by the formation of an inactive complex with the molecule or alternatively by the upregulation of inhibitory I $\kappa$ B. In the presence of stabilised mRNA transcripts for the inflammatory mediators, it is unlikely that such an effect on the inhibition of transcription would be noticeable, in the time period examined in this study.

It is unfortunate that in this study no significant decrease in mediator levels were detected following treatment with progesterone. It is clearly shown however, that all three mediators are rapidly induced or stabilised in response to FCS, PMA and even trauma from tissue collection and handling. Additionally, the limited response of IL-8, MCP-1 and COX-2 in secretory endometrium to induction by serum and PMA indicates an effect of endogenous progesterone in inhibiting mediator expression. To learn more about the mechanism involved in the down regulation observed in other systems, the above described methodological problems need to be overcome, to develop a model in which a progesterone effect can be studied effectively *in vitro*.

## **CHAPTER 6**

### **LOCALISATION AND TEMPORAL EXPRESSION OF PROLACTIN RECEPTOR IN HUMAN ENDOMETRIUM**

## 6.1 Introduction

Prolactin is a major product of the decidualised endometrium, synthesised solely by the decidualised stromal cells (Wu et al., 1995). This is also true for pseudodecidualised stromal cells which differentiate during the mid-late secretory phase of the menstrual cycle, and *in vitro* decidualisation of oestradiol primed stromal cells with progesterone results in prolactin expression (Daly et al., 1983). Indeed, prolactin is commonly used as a marker for functional decidualisation.

Prolactin has numerous functions in tissues around the body, as described in more detail in the general introduction (section 1.6). Possible functions of interest with respect to uterine function are its roles in immunomodulation, cell proliferation and differentiation and osmoregulation (Chilton, 1985; Healy, 1985; Draca, 1995). In the peripheral circulation, prolactin acts via the membrane bound receptor (PRL-R) to stimulate a wide range of leukocyte functions, including the proliferation of leukocytes previously stimulated with mitogenic factors, including interleukin-2 (IL-2) and T and B cell mitogens (Clevenger et al., 1990). An immunosuppressive action has also been indicated by the reduced immune response in lactating or PRL treated castrated rats (Dineen et al., 1972; Kelly et al., 1973). Prolactin has a unique function in initiating lactation in the mammary glands, stimulating the development of glandular components during pregnancy and in the regulation of milk production (Houdebine et al., 1985). Finally, a prolactin receptor defect in the amniotic epithelium of the amniochorion has been linked with the incidence of polyhydramnios, supporting a role in the regulation of amniotic fluid volume (Healy, 1985).

The role that prolactin plays in the endometrium is uncertain. Specific binding sites for the locally produced hormone have been identified in the amniochorion, third trimester decidua and in the decidua capsularis surrounding the first trimester fetal membranes (Healy, 1985; Tadokoro et al., 1995; Maaskant et al., 1996). However, a conclusive investigation into the spatial localisation of the receptors in non-pregnant and early pregnant decidua (decidua parietalis, lining the uterus) has not been conducted.

This study aims to examine prolactin receptor expression in the endometrium by immunolocalisation of the protein and RT-PCR to determine the temporal expression of the mRNA transcripts. Particular emphasis will be placed on the possible expression of the receptor by endometrial leukocytes with the aim to gain a greater



understanding of the functions that prolactin fulfils in the uterus. In addition, endometrium exposed to levonorgestrel (synthetic progestogen) via an intra-uterine system (Lng-IUS) shall be included in the study (Chapter 4, section 4.2). As previously described (Chapter 4, section 4.3), the presence of the steroid releasing system causes extensive decidualisation of the endometrium. The effect of extensive decidualisation on PRL-R expression and localisation shall therefore be examined.



**6.2 Materials and Methods**

Normal endometrial tissue at all stages of the menstrual cycle (n = 32) and first trimester decidua parietalis (n = 10) were investigated in this study (see table 11). In addition, endometrium exposed to progestogen via an Lng-IUS for 1, 3, 6 and 12 months (n = 14 patients), was examined for prolactin receptor expression (see chapter 4, section). Tissue samples were fixed in 10% NBF overnight and processed into paraffin blocks.

RNA was extracted from frozen control endometrium (proliferative n = 12, secretory n = 12) and first trimester decidua (n = 6) as described previously.

**Table 11** Table illustrating endometrial and decidual biopsies used in the present study.

STAGE OF CYCLE	DAYS FROM LMP	NUMBER OF BIOPSIES
Menstrual	d1-4	3
Proliferative	d5-13	9
Ovulatory	d14	3
Early Secretory	d15-18	4
Mid Secretory	d19-24	7
Late Secretory	d25-mens	6
First Trimester Decidua	8-10 weeks amenorrhoea	10

## **Immunohistochemistry**

Endometrium collected from all stages of the menstrual cycle, early pregnancy and in endometrium exposed to local Lng were examined for PRL-R immunoreactivity. Paraffin sections (5µm) were subjected to immunohistochemistry for prolactin receptor, using a rabbit polyclonal antibody raised against the highly conserved extracellular domain of the prolactin receptor. The immunostaining protocol is described in General Materials and Methods.

Human term fetal membranes were included as a positive control for the PRL-R immunohistochemistry (Maaskant et al, 1996). A matching concentration of non-immunised rabbit IgG was substituted for the primary antibody to exclude the possibility of non-specific binding.

## **Dual Immunohistochemistry**

Dual immunohistochemistry was utilised to investigate the relationship between prolactin receptor positive cells and endometrial leukocytes in representative secretory phase endometrium samples (n = 5). The method is described in general methods and materials, section 2.22.

## **Semi-quantitative RT-PCR**

Semi-quantitative RT-PCR was conducted to examine PRL-R mRNA transcript expression in human endometrium. Biotinylated antisense oligonucleotide primers complementary to the prolactin receptor were unavailable, meaning that quantitative ELISAs could not be conducted. The RNA samples were however subjected to amplification for GAP-DH to assess viability of RNA and to allow approximate equal loading of RNA template.

## **GAP-DH RT-PCR**

A sample of 400ng of total RNA (as calculated by optical density measurement, see section, 2.24) was included as the template for reverse transcription to cDNA. The detailed protocol is described in General Materials and Methods. Analysis of products by electrophoresis enabled assessment of viability of RNA and bands of comparable intensity indicated comparable RNA loading into the RT-PCR.

## **PRL-R RT-PCR**

Gene specific cDNA for PRL-R was generated by the reverse transcription of 400ng of total RNA from identical stocks as for GAP-DH. The total volume of reverse transcriptase products was subsequently amplified by PCR for 30 cycles to produce a 650bp product as described in General Materials and Methods (sections 2.25 - 2.26). The products were analysed by comparison with a molecular weight marker on a 1% agarose gel containing ethidium bromide. The bands were visualised on a UV transilluminator and photographed.

The identity of the RT-PCR product was confirmed by subcloning into expression vector pGEM®-T Easy Vector (Promega Ltd., Southampton, U.K.) and sequencing using the dideoxy chain termination method.

## **Southern Hybridisation**

The identity of the amplified PRL-R product was confirmed by Southern hybridisation (Southern, 1975). Representative RT-PCR products, amplified from RNA extracted at different stages throughout the menstrual cycle, were selected for analysis. The products were separated by electrophoresis on a 1% agarose gel and blotted overnight onto a nylon membrane by capillary transfer. Hybridisation was conducted overnight at 42°C with a 562bp cDNA, labelled with  $\alpha^{32}\text{P}[\text{dCTP}]$  by random priming, encoding the conserved extracellular domain (82% homology with human PRL receptor cDNA) of the red deer PRL receptor (between base pairs 96 and 657) (Clarke et al, 1995). Posthybridisation washes in SSC and SDS were conducted with increasing stringency and the membrane was thereafter exposed to autoradiographic film for 3 weeks.

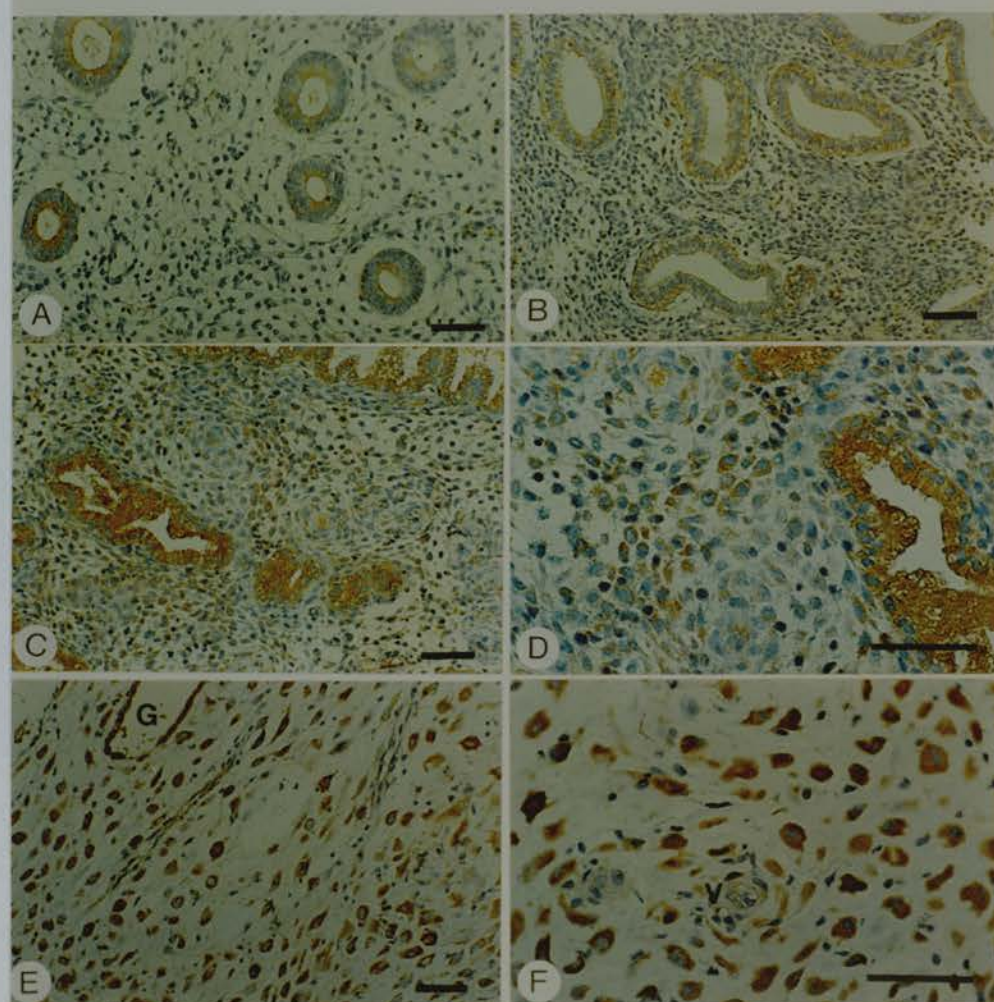
### 6.3 Results

Specific immunoreactivity for PRL receptor was detected in control endometrium and decidua by immunohistochemistry (Figure 58). In the proliferative and early secretory phases of the non-pregnant cycle, immunostaining was weak or absent in the stroma, with minimal immunoreactivity visible in the luminal region of the glandular epithelium. From the mid-late secretory phase and in early pregnancy, intense immunoreactivity was apparent in both the glands and a subpopulation of stromal cells. In addition, positive immunostaining was identified in the blood vessels in these stages.

Dual immunohistochemistry with an antibody recognising the leukocyte common antigen (LCA - CD45) did not conclusively demonstrate the presence or absence of PRL-R expression by endometrial leukocytes. A subset of CD45<sup>+</sup> cells appeared to exhibit immunostaining for PRL-R, but these were in the minority. Further, the majority of PRL-R<sup>+</sup> cells exhibited characteristics of large decidualised stromal cells. It is interesting to note however, that a portion of leukocytes present in blood contaminating the tissue section, as a result of biopsy collection, did possess immunoreactivity for PRL-R (not shown).

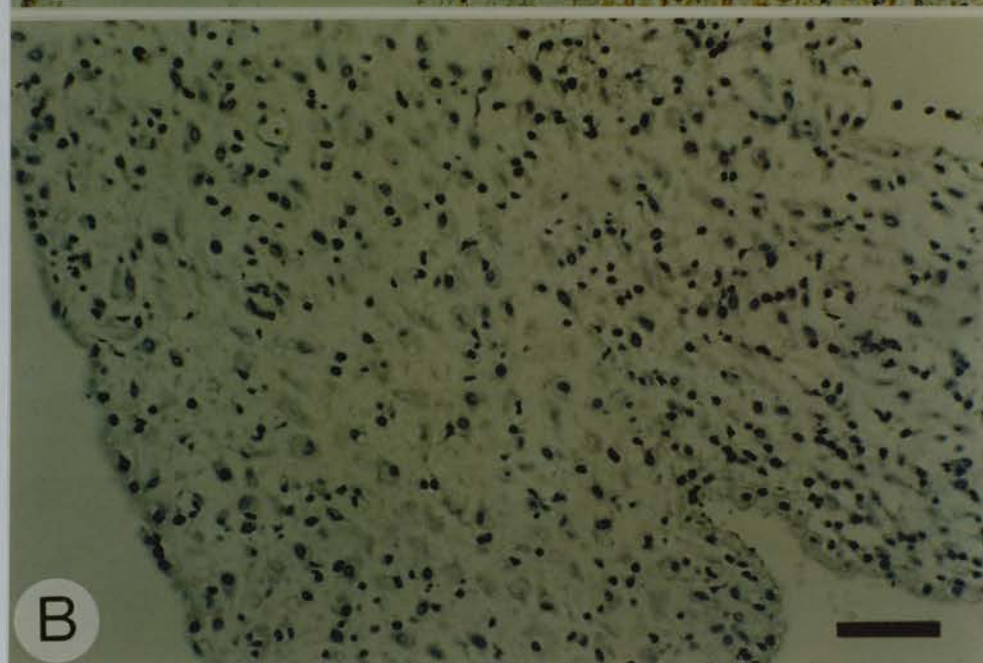
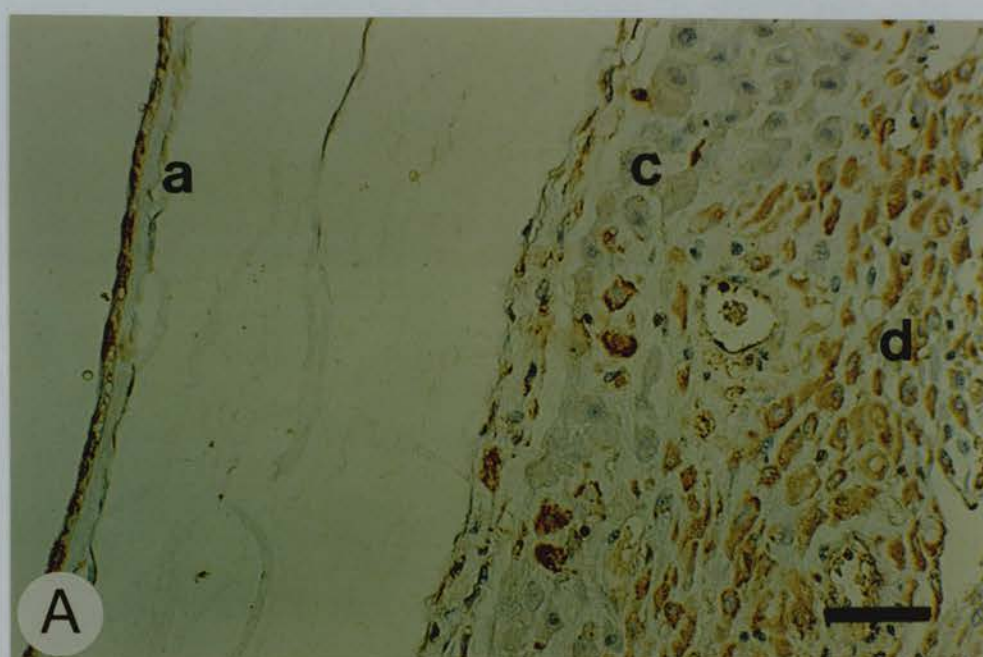
Fetal membranes included to act as a positive control exhibited positive immunoreactivity in the amniotic epithelium, chorion-laeve trophoblast and in the decidual layer as previously reported (Maaskant et al., 1996). Substitution of the primary antibody with rabbit IgG resulted in an absence of staining in fetal membranes, endometrial and decidual sections.

**Figure 58** Prolactin receptor immunolocalisation in human endometrium. Minimal immunoreactivity is observed in the proliferative phase (A) and early secretory phase (B) endometrium. In the mid-late secretory phase (C & D) intense immunostaining is present in the epithelial glands and a sub-population of stromal cells. This pattern persists in first trimester decidua (E) and at higher power (F) the decidualised stromal cells clearly exhibit strong immunoreactivity. (G = Glandular epithelium; V = vessel; Scale bars = 50µm)



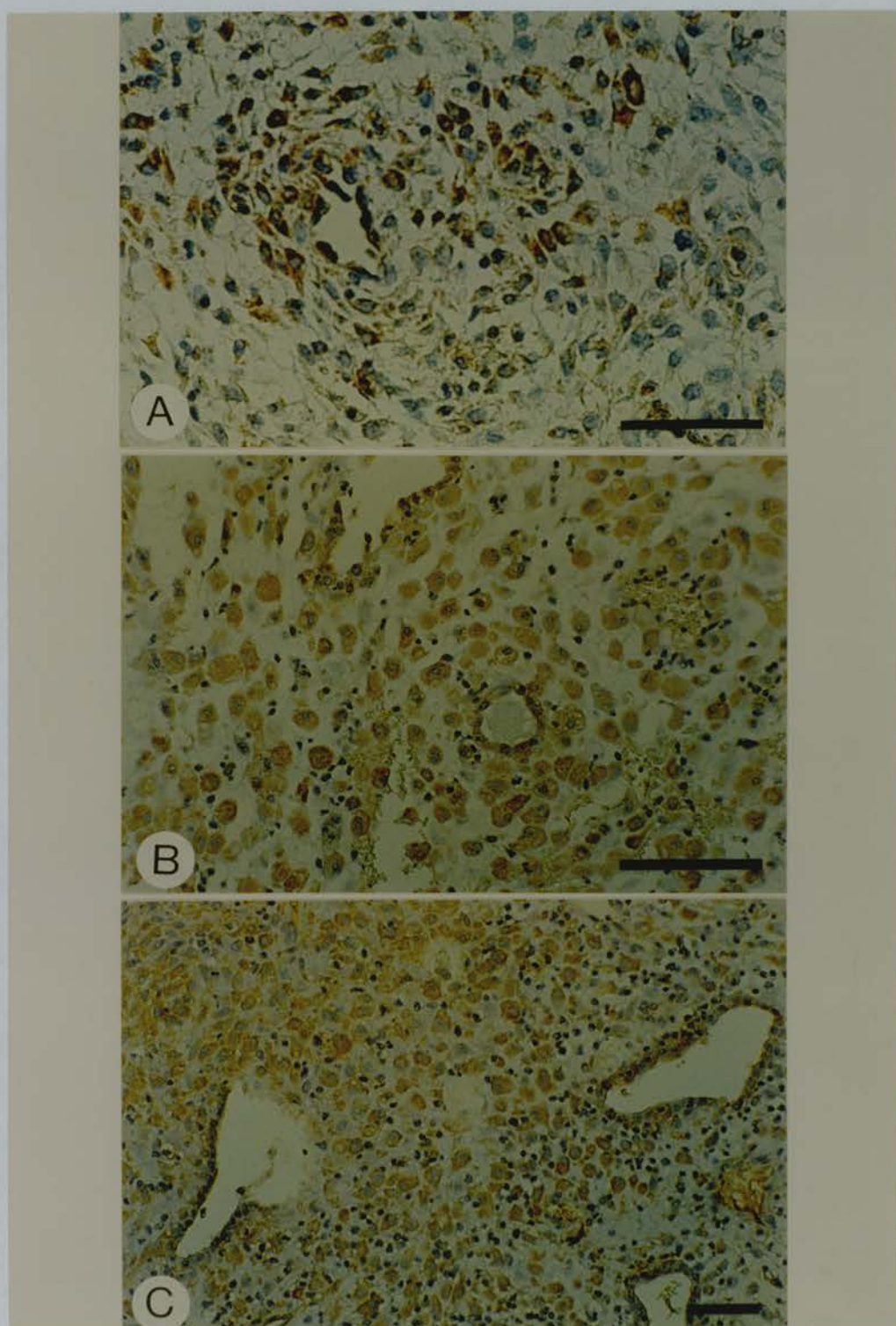
**Figure 59** Prolactin receptor immunoreactivity in human endometrium. (A) Term fetal membranes were included as a positive control and exhibited immunostaining in the amniotic epithelium, chorionic trophoblast and maternal decidua. (B) Rabbit immunoglobulins, at a matching concentration, were substituted for the primary antibody to act as a negative control, resulting in an absence of stain.





Progestogen exposed endometrium was also found to exhibit immunostaining for prolactin receptor. The presence of the Lng-IUS causes the endometrium to become extensively decidualised, as described in chapter 4 (section 4.3). The epithelial glands become atrophic and inactive, and stromal cells resemble large round decidualised cells with surrounding oedema (Silverberg et al., 1986; Luukkainen et al., 1990). Immunoreactivity in the pre-insertion endometrium was identical spatially and temporally to that observed in control endometrium. A significant increase ( $p < 0.05$ ) in glandular staining intensity was observed in the endometrium biopsied during the secretory phase compared to those from the proliferative phase. Glandular immunoreactivity remained high following insertion of the Lng-IUS ( $p < 0.05$  when compared to proliferative preinsertion). A significant increase in stromal immunostaining was detected postinsertion with respect to preinsertion proliferative ( $p < 0.05$ ) and secretory ( $p < 0.05$ ) phase immunostaining levels. The majority of the immunopositive stromal cells exhibited features of decidualised cells. Immunoreactivity was particularly pronounced in perivascular regions. A "patchy" pattern of staining was frequently observed in post-insertion endometrium, with areas of intense immunoreactivity and those with slight immunostaining within the same section. There were no significant differences in immunoreactivity at the different time points after insertion of the IUS or between postinsertion biopsies collected at different stages of the menstrual cycle.

**Figure 60** Prolactin receptor immunoreactivity in endometrium exposed to intra-uterine levonorgestrel. Immunostaining for PRL-R in preinsertion biopsies was consistent with the stage of the menstrual cycle, as described earlier. After insertion of the Lng-IUS, intense immunoreactivity was detected in glandular epithelium and in the decidualised stromal cells, particularly around blood vessels, at all time points. Photomicrograph (A) demonstrates immunoreactivity for PRL-R 1 month after insertion, (B) 3 months and (C) 6 months postinsertion.



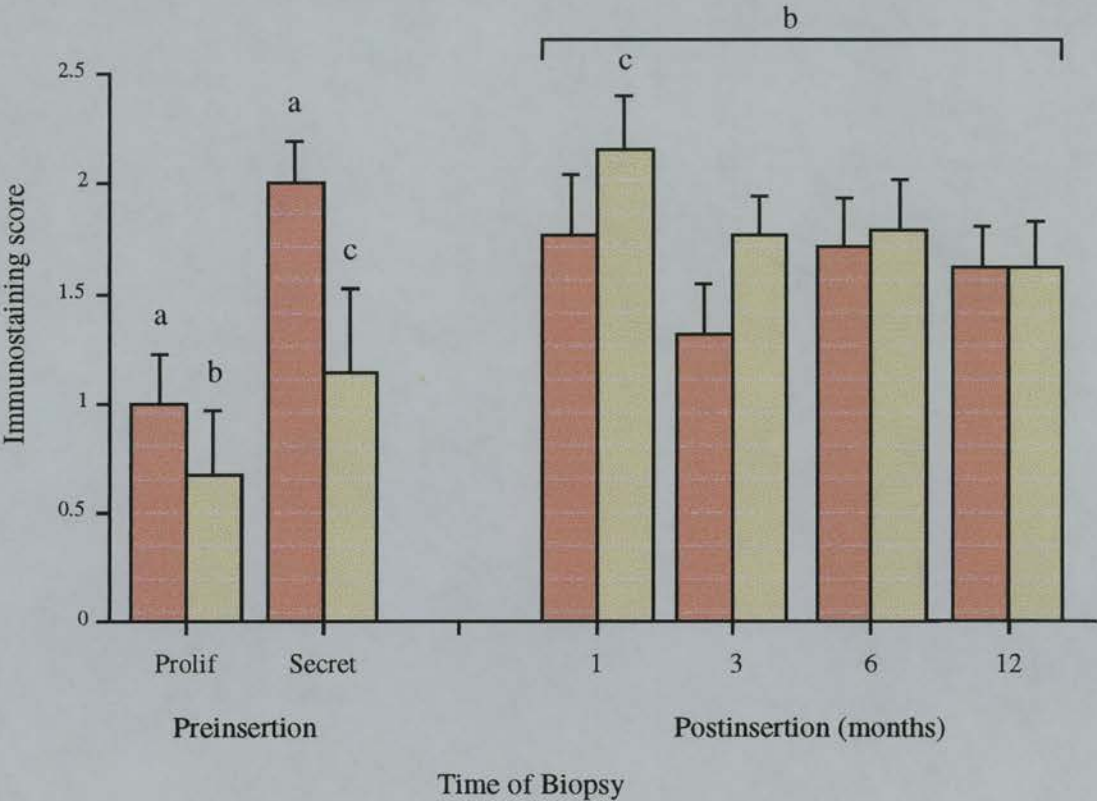


**Figure 61** Prolactin receptor immunoreactivity preinsertion and 1, 3, 6 and 12 months postinsertion of Lng-IUS. Stromal immunostaining is significantly elevated at 1, 3, 6 and 12 months postinsertion compared to preinsertion proliferative biopsies ( $p < 0.05$ ). A further elevation in stromal PRL-R immunoreactivity occurs between preinsertion secretory phase and 1 month postinsertion ( $p < 0.05$ ). Glandular immunoreactivity is increased from the proliferative to secretory phase preinsertion ( $p < 0.05$ ).

Figure 61

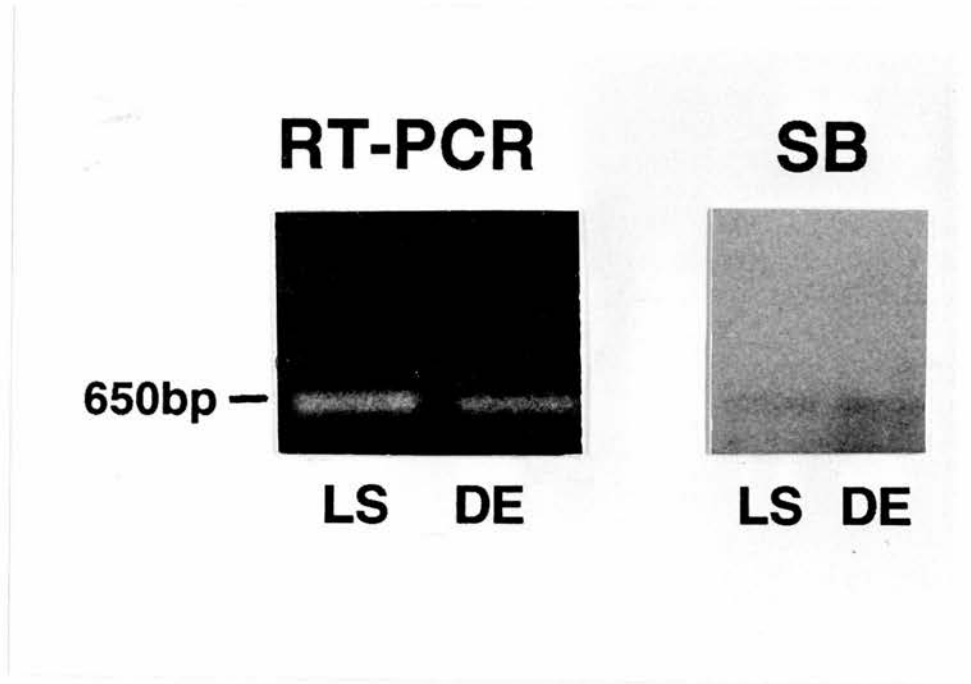
abc p < 0.05

Glands  
Stroma



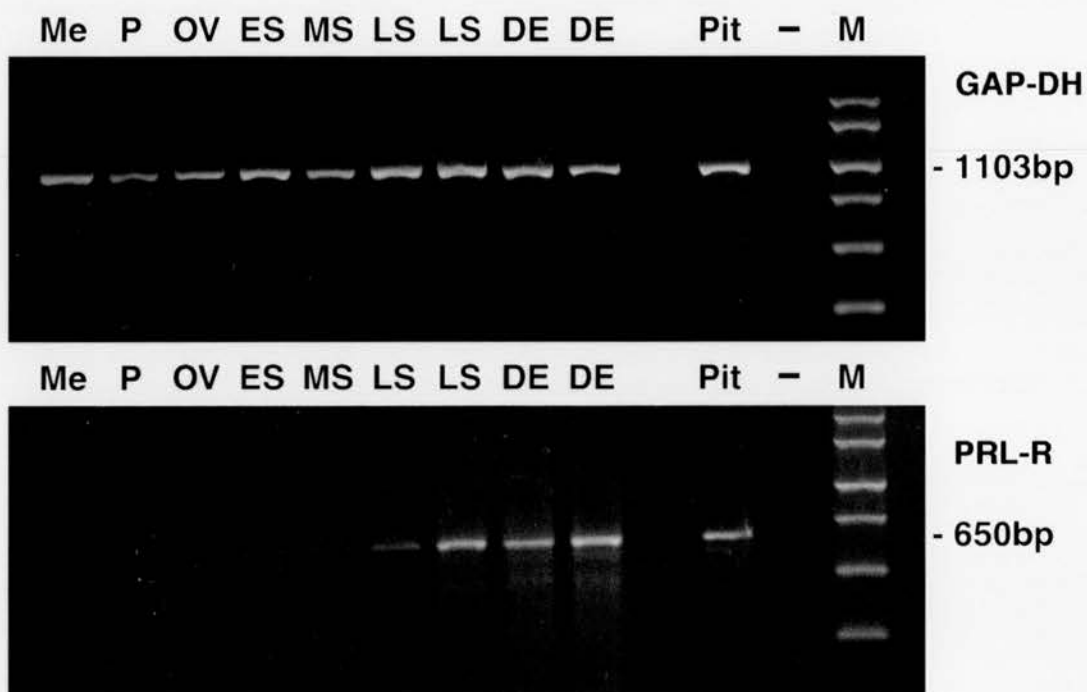
Following PRL-R RT-PCR, a single band of molecular weight 650bp was detected by gel electrophoresis, corresponding to the region of the PRL receptor gene amplified. Confirmation of its identity was obtained by Southern hybridisation (Figure 2); and sequence analysis verified that the amplicon encoded human PRL-R. A band representing PRL-R was detected in the tissue biopsied during the menstrual phase (d1-4). During the proliferative and early secretory stages, the signal was either weak or absent. From the mid to late secretory phase, however, a strong signal was detected, with increasing intensity in samples obtained from early pregnancy (Figure 3). A GAP-DH signal of comparable intensity (1103bp) was obtained from all samples, indicating that equivalent amounts of RNA were loaded into the RT-PCR. Furthermore, the presence of a GAP-DH signal in the absence of a PRL-R signal indicates that this was not due to an absence of viable RNA, but to the inability to detect PRL-R mRNA in the RNA sample. Overall, although this method is not totally quantitative, these data suggest that prolactin receptor expression is dramatically upregulated in the mid-late secretory phase and continues in early pregnancy.





**Figure 62** Southern hybridisation for PRL-R RT-PCR product. RT-PCR for PRL-R generated a 650bp amplified product, as identified by comparison with a molecular weight marker. The identity of the amplicon was confirmed by Southern Blotting (SB) and hybridisation with a cDNA probe raised against the extracellular domain. (LS = late secretory, DE = decidua).

**Figure 63** Temporal expression of PRL-R mRNA throughout the menstrual cycle. RNA extracted from tissues from each stage of the menstrual cycle and early pregnancy was subjected to RT-PCR to determine the temporal expression of PRL-R. The upper panel shows representative RNA samples amplified for GAP-DH (amplicon size = 1103bp), indicating the comparable loading of viable RNA into the prolactin receptor RT-PCR. The lower panel shows the same RNA samples amplified for PRL-R (amplicon size = 650bp). No signal was obtained in either the menstrual (Me), proliferative (P), ovulatory (OV) and early secretory (ES ) phases. From the mid and late secretory phase (MS / LS), a signal was achieved, increasing in intensity into early pregnancy (DE = decidua). Pituitary (Pit) RNA was included as a positive control, yielding a band of identical size. No signal was observed for the negative control (-).



## 6.4 Discussion

This study confirms the expression of the prolactin receptor in endometrium and decidua. Immunohistochemical localisation revealed that the PRL-R is strongly expressed by the glandular epithelium and stromal cells in pseudodecidualised and decidualised endometrium. Immunostaining was minimal or absent in proliferative and early secretory phase endometrium. This temporal pattern of expression was confirmed by the amplification of PRL-R mRNA by RT-PCR, with the detection of a strong signal from the mid to late secretory phase of the non-pregnant cycle and in early pregnancy. Thus, the temporal expression of the receptor appears to mirror that of PRL by the decidualised endometrium. A further increase in the number of immunopositive cells was detected in endometrium exposed to intrauterine levonorgestrel, correlating with extensive decidualisation of the stroma.

It seems likely that progesterone has a role in the regulation of PRL receptor expression (Jikihara et al., 1996). It is unlikely to be a direct regulation, however, as is the case for PRL, whose production is stimulated and maintained by the progesterone-mediated decidualisation of the endometrium (Markoff et al., 1983; Wu et al., 1990). Regulation via decidual derived factors (Gu et al., 1994) or even prolactin itself may be envisaged (Posner et al., 1975; Dijane et al., 1979; Amit et al., 1984; Muccioli et al., 1994). An indirect regulation by progesterone is firmly supported by the absence of progesterone receptors in the glandular epithelium in normal secretory phase endometrium, when PRL-Rs are expressed. This is further supported by the intense immunoreactivity observed in Lng exposed endometrium at a time when PgR are markedly downregulated (Critchley et al., 1998a) in both glandular epithelium and stroma.

The detection of strong immunoreactivity for the receptor in the epithelial glands suggest a paracrine action for PRL. During the early secretory phase, the endometrial glands become tortuous and actively produce uterine secretions, which are vital for preparing the endometrium for implantation (Noyes et al., 1950). During the decidualisation of the uterine stroma and continuing into pregnancy, the glandular epithelium becomes atrophic and glandular secretory activity ceases. This is also observed following local exposure to Lng. PRL has been reported to possess osmoregulatory activity, and the large amounts of PRL detectable in amniotic fluid act as a regulator of amniotic fluid volume. This was demonstrated by a link between defective PRL-R expression in amniotic epithelium and the incidence of

polyhydramnios (Healy, 1985). The presence of PRL receptors in the glandular epithelium during decidualisation implicates a similar role for PRL, that is in the downregulation of glandular activity.

Additional immunoreactivity was observed in a subset of stromal cells. The identity of these stromal cells is unknown, although the majority display morphological characteristics of decidualised stromal cells. This further supports the likelihood of the existence of an autocrine and / or paracrine interaction between PRL and its receptor, in decidual transformation. A role for prolactin in stimulating decidualisation, rather than being produced as a consequence of the transformation, is strongly reinforced by the multiple reproductive abnormalities displayed by the prolactin receptor knockout mouse, including failure of the endometrium to undergo decidualisation or implantation (Ormandy et al., 1996). Further co-localisation studies are required to determine whether the PRL producing cells concurrently express the receptor.

Prolactin interacts with its membrane bound receptor, a member of the haematopoietin / cytokine receptor superfamily, including growth hormone (GH), IL-2, IL-3, IL-4, IL-6, and LIF (Yu-Lee, 1997). Signal transduction occurs via interaction of the dimerised receptor with intracellular protein tyrosine kinases, including Janus kinase 1 (Jak1), 2 and 3. These phosphorylate intracellular regions of the PRL-R and cytoplasmic Stat proteins (signal transducer and activator of transcription). Phosphorylated and dimerised Stat proteins then translocate to the nucleus, where they are potent activators of target genes (Schindler et al., 1995). The specificity of the response is governed by the association of specific combinations of Jak and Stat proteins, which although is not fully understood, is believed to be determined by the amino acid sequence surrounding the phosphorylated tyrosine of the receptor, and by transcriptional regulation of the Jak and Stat proteins themselves. A clear involvement of Jak2 and Stat5 in prolactin signalling in the mammary gland has been demonstrated (Watson et al., 1996). A recent investigation by colleagues of the Jak/Stat pathway in the human endometrium has demonstrated that *in vitro* stimulation of isolated glandular and stromal elements by prolactin induces the phosphorylation of Jak2, and Stat-1 and -5, reinforcing the expression of fully functional PRL-R within the endometrium (Jabbour et al., 1997).

Extensive decidualisation of stromal cells is a characteristic of Lng exposed endometrium via an IUS. This correlates with elevated immunoreactivity for PRL-R in these cells, after one month with the Lng-IUS *in situ*. Continuous exposure to

Lng, over a period of 12 months, does not appear to alter the degree of PRL-R expression. A similar study investigating the immunoexpression of insulin-like growth factor binding protein-1 (IGFBP-1), a major product of decidualised cells (Bell, 1991), reported increased production of the binding protein after intra-uterine Lng delivery (Pekonen et al., 1992). Interestingly, they drew attention to the "patchy" nature of the immunostaining, relating to the degree of decidualisation and glandular activity. In the normal cycle, synthesis of IGFBP-1 is initiated when the first predecidual signs are apparent and increases with the progression of decidualisation (Bell, 1991). The effects of the Lng-IUS appear to be localised, endometrium in the direct vicinity of the device are more decidualised (Dallenbach-Hellweg, 1987). This explains the variations observed in immunoreactivity for PRL-R within a single tissue section.

Positive immunostaining for the receptor was detected in the vasculature of some decidualised endometrial samples examined. The perivascular cells frequently exhibited immunostaining as expected, as this is the site of initiation of decidualisation in the endometrium. Occasional staining was also observed that appeared to be endothelial in nature. A 16KDa N-terminal fragment of human PRL has been noted to be a potent inhibitor of angiogenesis (Clapp et al., 1993) and a high affinity binding site has been previously identified on endothelial cells (Clapp et al., 1992).

PRL has important immunoregulatory functions in the peripheral immune system, including leukocyte proliferation, differentiation and function. An important feature of the premenstrual endometrium and first trimester decidua is the accumulation of leukocytes within the decidualised stroma. These are believed to be essential for successful implantation and placentation (Loke et al., 1995b), but the mechanisms responsible for the premenstrual increase is not fully understood. The presence of an Lng-IUS in the uterus stimulates the accumulation of high numbers of leukocytes throughout the endometrium. Of particular interest are the uterine specific large granular lymphocytes (LGLs). It has been speculated that PRL may have a role in their proliferation and / or differentiation, especially after the discovery that these cells although clearly under the influence of progesterone (Inoue et al., 1996), do not express sex steroid receptors (King et al., 1996b). The effect of prolactin treatment on these decidual lymphocytes has been examined (Inoue et al., 1996; King et al., 1996b), but both studies failed to observe a resultant proliferation or differentiation. It is not unlikely, however, that prolactin may have an effect on decidual leukocytes, acting indirectly through autocrine / paracrine factors produced by neighbouring



cells. The failure of this study to clearly demonstrate the expression of prolactin receptors by the endometrial leukocytes may be due to the use of two enzyme detection systems. This introduces problems, as both protocols result in cytoplasmic immunostaining of target cells. A further approach may be to isolate the LGLs from the endometrium or use fluorescent detection system. It has recently been demonstrated, however, that the prolactin receptor is downregulated following stimulation in the prolactin dependant Nb2 T cell line (Elgberg et al., 1990), thought to be due to internalisation of the receptor-ligand complex or a decrease in mRNA levels (Clevenger et al., 1990; Perrot-Applanat et al., 1997).

It is probable that migration of specific leukocytes from the peripheral circulation also contributes to the increase in leukocyte numbers. This is supported by the elevated levels of the chemokines interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1), in the endometrium during leukocyte accumulation (Jones et al., 1997). Prolactin may be involved indirectly in enhancing this recruitment, as recent data reveals that the production of MCP-1 is stimulated by prolactin in ovarian tissue (Bowen et al., 1996). A number of roles for prolactin in the immunomodulation of the uterine leukocytes can thus be visualised, with a potential for augmenting lymphocyte migration, differentiation and proliferation.

In third trimester amniochorion, PRL has been implicated in the enhancement of matrix metalloproteinase (MMP) activity (Koay et al., 1986; Maaskant et al., 1996). During menses and placentation in early pregnancy, a dramatic reorganisation of extracellular matrix must occur to allow endometrial shedding and trophoblast invasion respectively. These processes invariably involve the action of MMPs, and it is possible that prolactin may participate as one of the factors capable of altering the activity of these enzymes in the endometrium.

In summary, this study clearly demonstrates the expression of prolactin receptors in the decidualised non-pregnant and pregnant endometrium, with a similar temporal pattern to prolactin. Immunohistochemical studies have isolated the glands and a subset of stromal cells as sites of action for prolactin. Prolactin has multiple functions throughout the body, including roles in growth, differentiation and immune response and it is therefore difficult to isolate a single function for PRL in the uterus. It is, however, likely that prolactin plays an important role in immunoregulation and additionally in the decidualisation of both the stromal and glandular cells of the endometrium. The increased expression of PRL-R corresponding to extensive decidualisation in endometrium locally exposed to synthetic progesterone further



supports this hypothesis.

## **CHAPTER 7**

### **GENERAL DISCUSSION**

## 7.1 Expression of Inflammatory Mediators in Human Endometrium

This thesis describes and reports findings concerning the expression of immunomodulatory and inflammatory agents in the human endometrium. The expression of chemokines, IL-8 and MCP-1, in human endometrium was examined. Immunoreactive protein was localised to the perivascular cells of all blood vessel types in non-pregnant and pregnant endometrium. This is consistent with a role in leukocyte recruitment and migration from the peripheral circulation into the endometrium.

Marked variations in intensity of immunoreactivity were detected at different stages of the menstrual cycle, most notably, with a reduction in immunostaining in the ovulatory and early secretory phases, followed by a significant elevation in the late secretory / premenstrual phase. IL-8 was additionally reduced in first trimester decidua, whilst strong MCP-1 immunoreactivity persisted. This temporal pattern was confirmed by monitoring mRNA expression levels using semi-quantitative RT-PCR. The upregulation of the chemokines in the premenstrual phase coincides with leukocyte accumulation in the endometrial stroma. In particular, IL-8 was strongly expressed in the premenstrual phase when an increase in the number of neutrophils has been reported. The high levels of MCP-1 protein and mRNA expressed in early pregnancy, corresponds to a continued elevation in the numbers of LGLs and macrophages. This temporal expression pattern is indicative of a negative regulation by the steroid hormone progesterone, as upregulation of IL-8 and MCP-1 mRNA and protein occurs in the premenstrual phase when concentrations of progesterone markedly decline.

Using a model of progesterone withdrawal and maintenance *in vivo*, it was demonstrated that IL-8 mRNA expression was upregulated following the withdrawal of exogenous progesterone and suppressed when endogenous progesterone was maintained. In contrast, MCP-1 mRNA was expressed in a progesterone environment, although a non-significant elevation was detected after exogenous steroid withdrawal. This is further evidence for the downregulation of IL-8 by progesterone in the human endometrium and is consistent with the anti-inflammatory actions of progesterone. MCP-1 also appears to be negatively regulated by progesterone, but to a lesser extent as the chemokine persists at high levels during progesterone dominated pregnancy. It is important to note that MCP-1 is differentially regulated to IL-8. In particular, MCP-1 is upregulated in response to

paracrine factors IL-4, LIF and PRL, all of which are prevalent during the mid secretory phase and first trimester decidua.

The inducible isoform of cyclooxygenase, COX-2, was detected by immunohistochemistry in the perivascular cells and in glandular epithelium. Again, weakest immunostaining was observed in the periovulatory phase, with significantly increased levels both premenstrually and menstrually. First trimester decidua examined possessed relatively low levels of expression of COX-2, although marked patient to patient variations were identified. COX-2 mRNA followed a similar expression pattern.

The localisation of COX-2 to the vasculature is consistent with the frequently described effects of prostaglandins in the modulation of vascular tone. Furthermore, the co-localisation of COX-2 with the chemokines is supportive of synergism between PGE (vasodilator) and IL-8 in neutrophil recruitment, and suggests a possible synergy with closely related MCP-1. Additional immunoreactivity in the glandular epithelium is in keeping with reports in the literature describing maximal secretion of prostaglandins by the glandular as opposed to the stromal components of the endometrium.

The expression patterns identified indicate lower production of prostaglandins during the early and mid secretory phases, when implantation may occur, and elevated production following withdrawal of progesterone in the absence of pregnancy. This is consistent with a regulated inflammatory type reaction occurring in the peri-implantation period, although this is obviously suppressed with respect to the amplified inflammatory reaction following steroid withdrawal premenstrually. An involvement of COX-2 in decidualisation and implantation is reinforced by the recent publication which investigated in great detail the reproductive failures associated with targeted disruption of the COX-2 gene in mice (Lim et al., 1997b). Deficiencies were described in the COX-2 *-/-* homozygous female during oocyte maturation and therefore also ovulation and fertilisation. Transferral of wild type blastocyst to recipient COX-2 *-/-* mice resulted in unsuccessful implantation, which is was attributed to the failure of the endometrium to pseudodecidualise following artificial stimulation. These findings were reinforced by the generation of a similar phenotype by the administration of a selective COX-2 inhibitor and detection of COX-2 mRNA expression by *in situ* hybridisation in the wild type mouse peri-implantation uterus. Together these data are strong evidence for the involvement of tightly regulated COX-2 in normal endometrial functioning, particularly during

decidualisation and implantation.

Progesterone has previously been implicated in the regulation of prostaglandins. Whilst the steroid appears to increase the capacity of the tissue to produce PGs during the secretory phase, the full potential is not realised until withdrawal of progesterone premenstrually (Smith et al., 1988). Moreover, the main metabolising enzyme, PGDH, is positively regulated by progesterone, resulting in low local concentrations of PGs during the secretory phase and throughout pregnancy (Casey et al., 1980). The data presented here indicate an opposing regulation for COX-2, with a suppression by progesterone. Withdrawal of exogenous progesterone *in vivo* resulted in significant upregulation of COX-2 mRNA, whilst maintenance of progesterone concentrations inhibited COX-2 mRNA expression. Thus removal of the inhibitory effect of progesterone on COX-2 expression premenstrually triggers an inflammatory response, characterised by elevated production of prostaglandins, whilst PG synthesis is downregulated during early pregnancy mediated by progesterone suppression of COX-2.

IL-8 and COX-2 immunoexpression was examined in endometrium exposed to high local doses of a synthetic progestin, levonorgestrel, via an intra-uterine releasing system. Following insertion of the Lng-IUS, an initial rise in IL-8 expression was detected, with a decline at 3 and 6 months postinsertion. Similarly, COX-2 was present at high levels 1 and 3 months after insertion of the device, with a subsequent decrease observed at 6 and 12 months postinsertion. Breakthrough bleeding is associated with progestin-only contraception, typically more frequent during the first 6 months of use. The strong expression of IL-8 and COX-2 in the initial biopsies collected after insertion of the device indicates that these local inflammatory mediators may have a role in the breakthrough bleeding episodes. The mechanisms involved in their upregulation during this period is uncertain, although it is noteworthy that expression of PgRs by both glandular and stromal compartments is suppressed during the first 6 months of exposure to Lng. Moreover, the Lng-IUS had been reported to dramatically reduce the amount of menstrual blood loss, thus proving to be a useful clinical management of menorrhagia. Prostaglandins are known to have a role in regulating vasculature tone during menstruation, and the reduction of COX-2 expression observed after 6 months of exposure to intrauterine Lng may be a contributory factor to the reduced blood loss reported.

Pharmacological withdrawal of progesterone via antigestogen (RU486) administration during first trimester pregnancy had no significant effect on



chemokine and COX-2 expression as assessed by immunohistochemistry, although transient elevations of immunoreactive IL-8 and MCP-1 were detected by ELISA. Blockade of the receptor is likely to result in a much more rapid downregulation of progesterone than is observed with the gradual decline of progesterone production premenstrually. Therefore, any changes may not have been identifiable at the time points examined.

Using an in vitro culture system, IL-8, MCP-1 and COX-2 mRNA could be induced in response to trauma, fetal calf serum and phorbol ester. Variations in the degree of stimulation were apparent, consistent with previous measurements of mediator expression during the normal menstrual cycle. In particular, tissue collected during the secretory phase had a lower capacity to synthesise mRNA for IL-8, MCP-1 and COX-2. This indicates that there may exist an intrinsic inhibitory mechanism for inflammatory mediator expression by secretory phase endometrium, possibly mediated through progesterone. Alternatively, the upregulation of mRNA expression may be a consequence of increased stability of existing mRNA transcripts, rather than *de novo* transcription. Thus the reduced stimulation of chemokines and COX-2 would reflect the low number of transcripts identified consistently throughout the secretory phase.

No significant changes in expression levels were detectable following exposure to progesterone for 2 hours. This is likely to be due to the short time span of exposure to the steroid. Progesterone has been described to interact with the upstream promoter sites through its transcription factor receptor, to directly inhibit transcription. If the increase observed in the presence of serum is increasing the stability of the normally transiently expressed mediators, then an inhibition of transcription would effectively be masked. The majority of COX-2 mRNA would, by reference to immunohistochemical studies, be transcribed in the glandular epithelium. During the secretory phase, these cells do not possess PgR and thus treatment with the steroid would not be able to have a direct effect.

Prolactin is a major product of the decidualised endometrium, yet its site of action and function within the endometrium is unknown. Prolactin receptors were localised by immunohistochemistry to the glandular epithelium and a subset of stromal cells, the majority of which exhibited characteristics of large decidualised stromal cells. This temporal expression pattern was confirmed by semi-quantitative RT-PCR, and mimics that of the ligand prolactin by the endometrium. Local exposure of the endometrium to levonorgestrel stimulates extensive decidualisation, reminiscent of

early pregnancy. This results in a significant increase in the number of PRL-R positive stromal cells. The expression of both ligand and receptor by the decidualised stromal cells indicates an autocrine loop in promoting decidualisation. This is supported by the recently described prolactin receptor knockout mouse (Ormandy et al., 1996). The homozygous mouse deficient for prolactin receptor was infertile, due to failed blastocyst implantation. The endometrium did not undergo decidualisation, and thus implantation was unsuccessful. The identification of the epithelial glands as a target for prolactin suggests a role for prolactin in the regulation of glandular activity.

It was not possible to identify receptor expression on specific leukocyte populations, although blood contaminating tissue sections frequently included leukocytes strongly positive for the prolactin receptor, in line with its function as a potent immunomodulatory agent in the peripheral immune system. It remains a possibility that PRL may have a stimulatory action on endometrial leukocytes.

## **7.2 Roles for Locally Acting Mediators in Preparation of the Endometrium for Implantation**

The endometrium undergoes decidualisation in the mid-late luteal phase to provide a suitable environment for implantation. Stromal cells differentiate and become enlarged, glandular activity is reduced and an accumulation of leukocytes is apparent in the oedematous stroma. These changes are mediated by progesterone, likely to be acting indirectly via expression of local mediators.

PRL is likely to act in an autocrine / paracrine manner to stimulate decidual changes in stromal and glandular elements. PGE<sub>2</sub> has been linked with the vasodilation which is a necessary prerequisite for decidualisation, and in facilitating the decidual cell reaction (Kennedy et al., 1981; Kennedy, 1986). COX-2 expression would appear to be essential for the production of PGs in the peri-implantation uterus (Lim et al., 1997b). Furthermore, in the uterus, PGE<sub>2</sub> is upregulated by PRL through elevated expression of COX-2 (Prigent-Tessier et al., 1996).

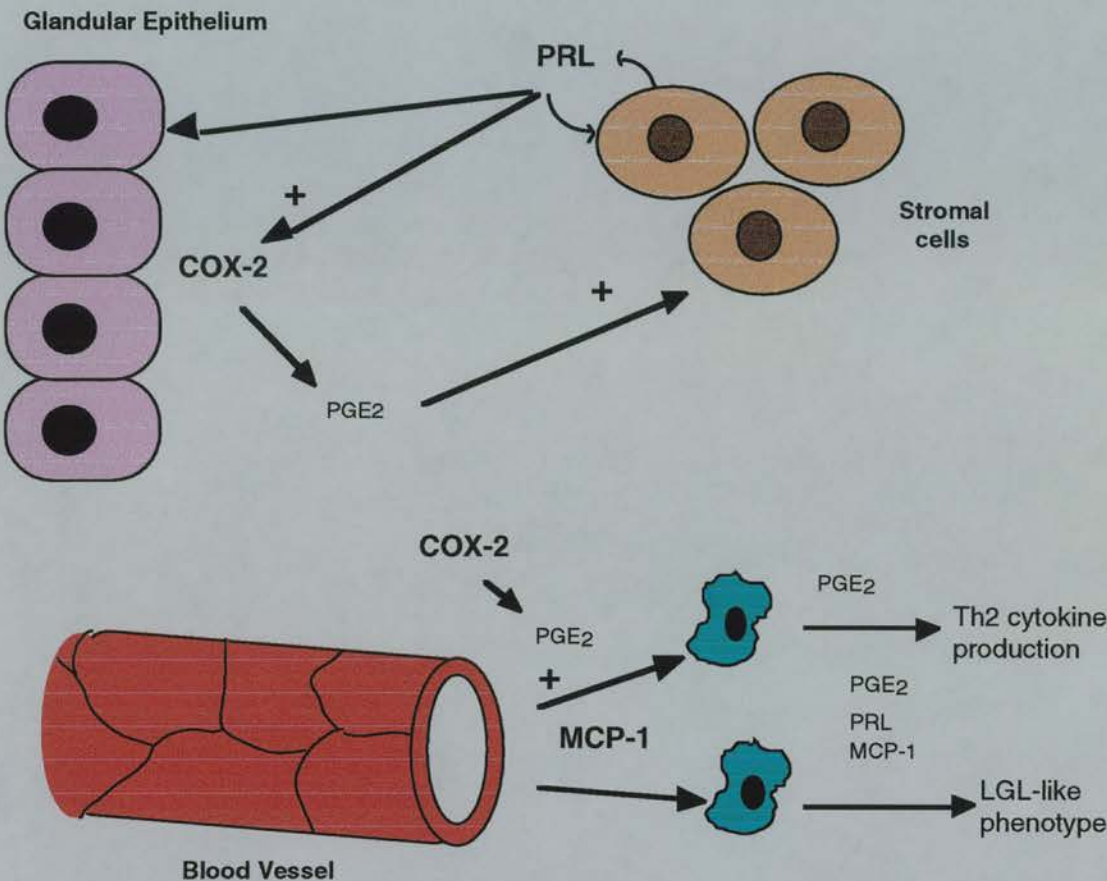
MCP-1 expression in a perivascular location is appropriate for a role in leukocyte recruitment, and co-localisation with COX-2 supports the opportunity for a synergism between PGE and MCP-1. In addition, PRL has been described to stimulate MCP-1 expression in the ovary, thereby augmenting leukocyte recruitment (Bowen et al., 1996). A number of locally acting factors, including MCP-1, PRL and PGE<sub>2</sub> (Linnemeyer et al., 1993), have been demonstrated to activate NK cells,



potentially to induce an LGL-like phenotype. Thus together, the effect on the endometrium would be decidualisation of the stroma and glands, vasodilation producing oedema and leukocyte infiltration. The co-expression of the three local mediators, PRL, MCP-1 and COX-2, suggests interactions in the endometrium to promote decidualisation and implantation.

There is evidence that production of Th2-type cytokines by endometrial T cells and macrophages is favourable for pregnancy (Wegmann et al., 1993, Lim et al., 1998). A number of Th2 cytokines have been detected in the maternal-fetal unit, where a humoral response as opposed to an inflammatory response is favoured. PGE<sub>2</sub> can stimulate a switch from inflammatory cytokine production (Th1) to a humoral immune response (Th2 cytokine profile) (Snijdwint et al., 1993; Strassmann et al., 1994; Hilkens et al., 1995; Kraan et al., 1995, Huang et al., 1998). The balance of Th1 and Th2 cytokines regulates chemokine expression, with MCP-1 preferentially upregulated by Th2 type cytokines (IL-4, IL-10) (Rollins et al., 1991; Gu et al., 1997) and in Th2 type situations (Chensue et al., 1996; Warmington et al., 1996). Moreover, expression of MCP-1 appears to induce a Th2 response (Gu et al., 1997), through the differentiation of naive T cells to Th2 cells (Karpus et al., 1997a; Karpus et al., 1997b) and upregulation of IL-4 (Lukacs et al., 1997), whilst downregulating Th1 cytokine IL-12 (Chensue et al., 1996). This may also be the case for other CC chemokines, as gene targeting to knock-out the CCR1 receptor (ligands include MCP-3, MIP-1 $\alpha$  and RANTES), resulted in impaired host defence when challenged and a bias towards Th1 cytokine production (Gao et al., 1997). In contrast, IL-8 is generally inhibited by Th2 cytokines (Standiford et al., 1990; de Waal Malefyt et al., 1991; John et al., 1998) and upregulated in inflammatory situations characterised by Th1 cytokine expression (Edwards et al., 1997). Expression of COX-2, consistent with its involvement in inflammatory response, is downregulated by IL-10 and IL-4 (Mertz et al., 1994; Niirio et al., 1997). The expression patterns of inflammatory mediators within the endometrium reported in this thesis, i.e. reduced expression of IL-8 and COX-2 during pregnancy, and elevated MCP-1, are therefore in agreement with a tilt towards Th2 cytokine production during pregnancy.

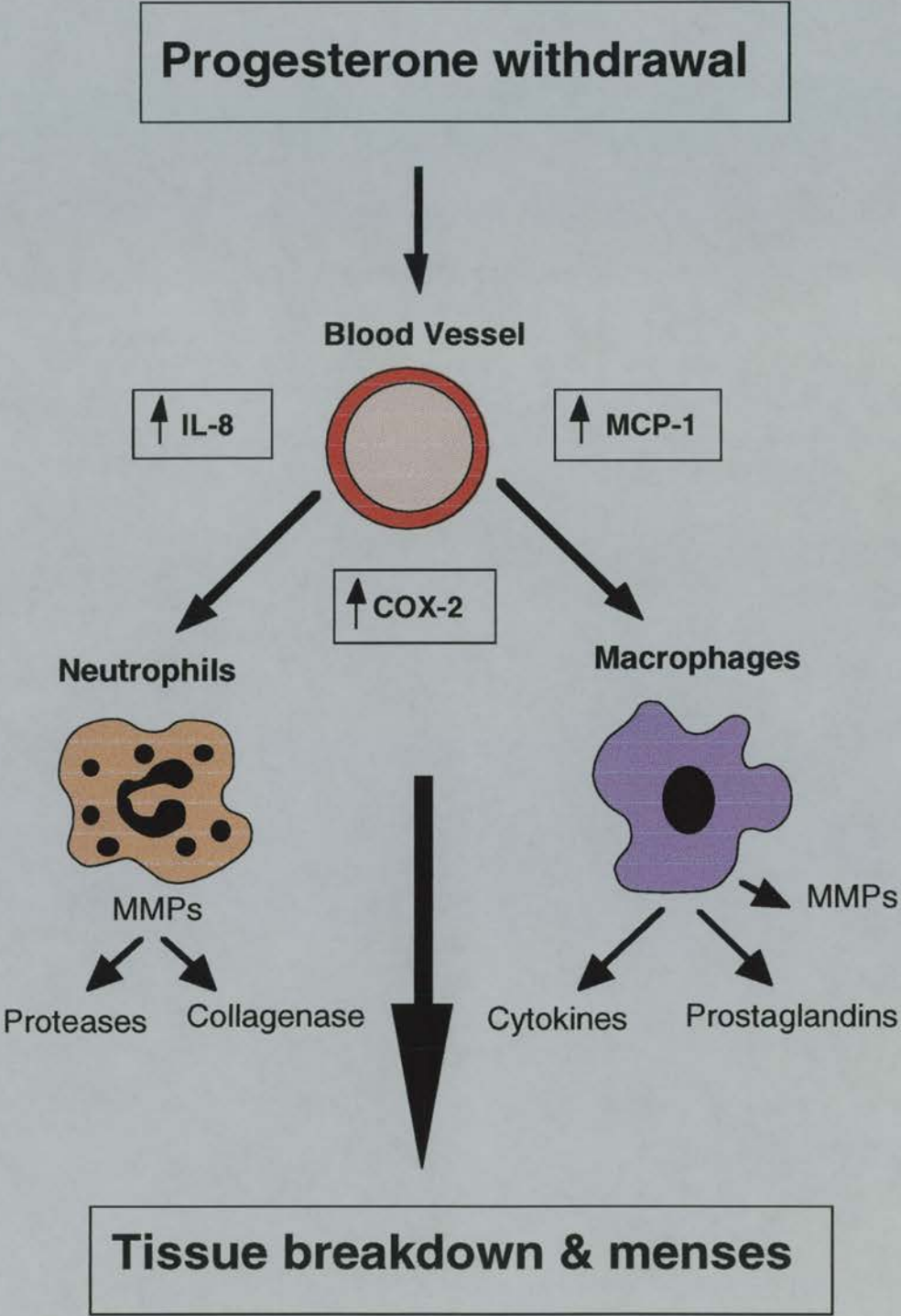
Figure 65



### **7.3 Involvement of Inflammatory Mediators in the Initiation of Menstruation**

The fall in progesterone concentrations due to regression of the corpus luteum initiates the breakdown and shedding of the endometrium during menstruation. This process in many ways resembles an inflammatory response, with increased vascular permeability, accumulation of neutrophils and elevated prostaglandin production. The strong expression of COX-2 in the perimenstrual period reinforces a role for the inducible isoform in normal endometrial physiology. The elevated expression of IL-8 and MCP-1 at a time when leukocyte numbers increase is strongly supportive of the hypothesis that leukocytes migrate from the peripheral circulation. All three inflammatory mediators are to some degree downregulated by progesterone, thus the premenstrual withdrawal of progesterone would stimulate an increase in expression of both mRNA and functional protein. This conceivably could result in leukocyte infiltration, accompanied and possibly facilitated by vasodilation mediated through prostaglandins. Simultaneous with the upregulation of COX-2, the metabolising enzyme PGDH is suppressed by progesterone withdrawal, resulting in high local concentrations of PGs. Leukocytes are potent sources of MMPs and cytokines, together capable of degrading the components of the endometrium and amplifying an inflammatory response. The chemokines and COX-2, through the production of inflammatory prostaglandins may therefore be envisaged to have major role in the inflammatory reaction that characterises menstruation.

Figure 66



## 7.4 Future Studies

This thesis provides strong evidence for leukocyte recruitment to the endometrium, directed by chemokines, contributing to the leukocyte increase observed in the mid-late secretory phase. The mechanisms involved in the appearance of the LGLs is of particular interest, and the temporal expression patterns described here along with evidence in the literature suggests that a precursor cell type (subset of circulating NK cells) may be recruited by MCP-1 to the endometrium. This is of prime interest within the field of endometrial immunology and this possible connection could be investigated in greater depth to confirm a relationship between circulating NK cells, chemokine and LGLs.

The strong expression of the chemokines and COX-2 immediately before and during menses is consistent with the inflammatory type response which characterises menstruation and confirms the importance of these inflammatory mediators in the process of menstruation. An obvious continuation of these studies would be to examine the expression of the chemokines and COX-2 in endometrium collected from women experiencing dysfunctional menstrual bleeding. The aetiology of menstrual disorders such as menorrhagia (uncomplicated by organic or endocrine disorders) and inter-menstrual bleeding caused by progestin-only contraceptives is not known (Cameron, 1989). There is evidence for elevated production of vasodilatory prostaglandins in endometrium from women with menorrhagia (Cameron et al., 1986b) and the preliminary evidence provided in the present study for the expression of IL-8 and COX-2 in Lng exposed endometrium reinforces a role for these local mediators in the breakthrough bleeding episodes, and similarly, the chemokines have been linked with the inflammatory condition endometriosis.

These studies contribute to the understanding of the paracrine interactions within the endometrium, and it is conceivable in the future that knowledge of normal endometrial function will lead to development of non-hormonal methods of contraception, fertility treatment or treatment of menstrual disorders (Nie et al., 1997). In the recent years, much interest has been placed on the development of chemokine antagonists (Wells et al., 1996a; Wells et al., 1996b), with emphasis placed on preventing infection with HIV, for which CC chemokine receptors are a cell entry co-factor (Feng et al., 1996). This technology may also have a role in the reproductive system and other inflammatory situations (Harada et al., 1996).

Since the discovery of the inducible isoform of cyclooxygenase (COX-2) and its



identification as the isoform predominantly expressed in inflammatory situations, work has been conducted to develop non steroidal anti-inflammatory agents (NSAIDs) which selectively target COX-2, whilst leaving COX-1 unaffected, thus avoiding the gastrointestinal side effects (Bennett et al., 1995; Vane et al., 1995). A number of compounds, nimesulide, NS-398 and Dup-697 all preferentially inhibit COX-2 activity (Futaki et al., 1994; Gierse et al., 1995; Tarvares et al., 1995), and an example of the potential of such substances is the case study presented earlier this year where preterm labour was delayed by administration of nimesulide (Sawdy et al., 1997). The strong expression of COX-2 in the perimenstrual period indicate that a selective COX-2 inhibitor may have a role in treating menorrhagia or dysmenorrhoea.

## BIBLIOGRAPHY

- Abberton, K. M., Taylor, N. H., Healy, D. L. and Rogers, P. A. W. (1996). Vascular smooth muscle  $\alpha$ -actin distribution around endometrial arterioles during the menstrual cycle: increased expression during the perimenopause and lack of correlation with menorrhagia. *Hum Reprod* **11** : 204-211.
- Abel, M. H. and Baird, D. T. (1980). The effect of  $17\beta$  estradiol and progesterone on prostaglandin production by human endometrium maintained in organ culture. *Endocrinology* **106** : 1599 - 1606.
- Adams, D. H. and Shaw, S. (1994). Leukocyte-endothelial interactions and the regulation of leucocyte migration. *Lancet* **343** : 831-836.
- Akoum, A., Lemay, A., Brunet, C. and Hebert, J. (1995). Cytokine-induced secretion of monocyte chemotactic protein-1 by human endometrial cells in culture. *Am J Obstet Gynecol* **172** : 594-600.
- Akoum, A., Lemay, A., McColl, S., Turcot-Lemay, L. and Maheux, R. (1996). Elevated concentration and biologic activity of monocyte chemotactic protein-1 in the peritoneal fluid of patients with endometriosis. *Fertil Steril* **66** : 17-23.
- Alam, R. (1997). Chemokines in allergic inflammation. *J Allergy Clin Immunol* **99** : 273-277.
- Allavena, P., Paganin, C., Martin-Padura, I., Peri, G., Gaboli, M., Djana, E., Marchisio, P. C. and Mantovani, A. (1992). Molecules and structures involved in the adhesion of natural killer cells to vascular endothelium. *J Exp Med* **173** : 439-448.
- Allavena, P., Bianchi, G., Zhou, D., van Damme, J., Jilek, P., Sozzani, S. and Mantovani, A. (1994). Induction of natural-killer-cell migration by monocyte chemotactic protein-1, protein-2 and protein-3. *Eur J Immunol* **24** : 3233-3236.
- Amit, T., Barkey, R. J., Garvish, M. and Youdim, M. B. H. (1984). Induction of prolactin (PRL) receptors by PRL in the rat lung and liver - demonstration and characterisation of a soluble receptor. *Endocrinology* **114** : 545-552.
- Andersson, K. and Rybo, G. (1990). Levonorgestrel-releasing intrauterine device in the treatment of menorrhagia. *Br J Obstet Gynaecol* **97** : 690-694.



Arenberg, D. A., Polverini, P. J., Kunkel, S. L., Shanafelt, S. L., Hesselgesser, J., Horuk, R. and Strieter, R. M. (1997). The role of CXC chemokines in the regulation of angiogenesis in non-small cell lung cancer. *J Leuk Biol* **62** : 554-562.

Arici, A., Head, J. R., MacDonald, P. C. and Casey, M. L. (1993). Regulation of interleukin-8 gene expression in human endometrial cells in culture. *Mol Cell Endocrinol* **94** : 195-204.

Arici, A., MacDonald, P. C. and Casey, M. L. (1995). Regulation of monocyte chemotactic protein-1 gene expression in human endometrial cells in culture. *Mol Cell Endocrinol* **107** : 189-197.

Arici, S., MacDonald, P. C. and Casey, M. L. (1996). Modulation of the levels of interleukin-8 messenger ribonucleic acid and interleukin-8 protein synthesis in human endometrial stromal cells by transforming growth factor- $\beta$ 1. *J Clin Endocrinol Metab* **81** : 3004-3009.

Arici, A., Oral, E., Bukulmez, O., Buradagunta, S., Bahtiyar, O. and Jones, E. E. (1997). Monocyte chemotactic protein-1 expression in human preovulatory follicles and ovarian cells. *J Reprod Immunol* **32** : 201-219.

Austgulen, R., Lun, E., Liabakk, N., Jacobsen, G. and Arntzen, K. J. (1994). Increased levels of cytokines and cytokine activity modifiers in pregnancy. *Eur J Obstet Gynaecol Reprod Biol* **57** : 149-155.

Baggiolini, M., Dewald, B. and Moser, B. (1994). Interleukin-8 and related chemotactic cytokines-CXC and CC chemokines. *Adv Immunol* **55** : 97-179.

Baggiolini, M., Dewald, B. and Moser, B. (1997). Human chemokines : an update. *Annu Rev Immunol* **15** : 675-705.

Bahzad, C., Wyssling, H., Saraya, L., Shi, Y., Prasad, R. N. V., Swahn, M. L., Kovacs, L., Belsey, E. M. and Van Look, P. (1989). Termination of early human pregnancy with RU486 (Mifepristone) and the prostaglandin analogue Sulprostone: a multicentre, randomized comparison between two treatment regimens. *Hum Reprod* **4** : 718-725.

Baird, D. T. (1993). Antigestogens. *Br Med Bull* **49** : 73-87.

Baird, D. T. (1993). Potential contraceptive effects of antigestogens. In (Eds) Donaldson, M., Dorflinger, L., Brown, S. S. and Benet, L. Z. *Clinical applications of Mifepristone (RU486) and other antiprogestins. Proc. of Institute of medicine, National Academy of Sciences: Committee on Antiprogestins: Assessing the Science.* National Academy Press, Washington : 148-163.

Baird, D. T., Cameron, S. T., Critchley, H. O. D., Drudy, T. A., Howe, A., Jones, R. L., Lea, R. G. and Kelly, R. W. (1996). Prostaglandins and menstruation. *Eur J Obstet Gynaecol Reprod Biol* **70** : 15-17.

Barbosa, I., Bakos, O., Olsson, S. E., Odland, V., Eloff, D. B. and Johansson (1990). Ovarian function during use of a levonorgestrel-releasing IUD. *Contraception* **42** : 51-66.

Barclay, C. G., Brennand, J. E., Kelly, R. W. and Calder, A. A. (1993). Interleukin-8 production by the human cervix. *Am J Obstet Gynecol* **169** : 625-632.

Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D. R., Zlotnik, A. and Schall, T. J. (1997). A new class of membrane bound chemokine with a CX(3)C motif. *Nature* **385** : 640-644.

Belin, D. (1996). The RNase protection assay. *Meth Mol Biol* **58** : 131-136.

Bell, S. C. (1991). The insulin-like growth factor binding proteins - the endometrium and decidua. *Ann N Y Acad Sci* **622** : 120-137.

Ben-Baruch, A., Xu, L. L., Young, P. R., Bengali, K., Oppenheim, J. J. and Wang, J. M. (1995). Monocyte chemotactic protein-3 (MCP-3) interacts with multiple leukocyte receptors - C-C CKR1, a receptor for macrophage inflammatory protein-1 $\alpha$  / RANTES, is also a functional receptor for MCP-3. *J Biol Chem* **270** : 22123-22128.

Ben-Jonathon, N., Mershon, J. L., Allen, D. L. and Steinmetz, R. W. (1996). Extrapituitary prolactin : distribution, regulation, functions and clinical aspects. *Endocr Rev* **17** : 639-669.

Bennett, A. and Tavares, I. A. (1995). NSAIDs, Cox-2 inhibitors, and the gut. *Lancet* **346** : 1105.

- Bevilacqua, E. M. A. F. and Abrahamsohn, P. A. (1988). Ultrastructure of trophoblast giant cell transformation during the invasive stage of implantation of the mouse embryo. *J Morphol* **198** : 341-51.
- Biesecker, L. G. and Emerson, S. G. (1993). Interleukin-6 is a component of human umbilical-cord serum and stimulates hematopoieses in embryonic stem cells *in vitro*. *Exp Hematol* **21** : 774-778.
- Bjorn, S. F., Hastrup, N., Lund, L. R., Dano, K., Larsen, J. F. and Pyke, C. (1997). Co-ordinated expression of MMP-2 and it's putative activator MT1-MMP, in human placentation. *Mol Hum Reprod* **3** : 713-723.
- Blatchely, F. R. and Poyser, N. L. (1974). The effect of oestrogen and progesterone on the release of prostaglandins from the uterus of the ovariectomised guinea-pig. *J Reprod Fertil* **40** : 205-209.
- Bouchard, P., Marraoui, J., Massai, M. R., Medale, D. A., De Ziegler, D., Perrot-Appanat, M., Frydman, R. and Bergeron, C. (1991). Immunocytochemical localisation of oestradiol and progesterone receptors in human endometrium: a tool to asses endometrial maturation. *Bailliere's Clin Obstet Gynaecol* **5** : 107-115.
- Bowen, J. M., Landis Keyes, P. L., Warren, J. S. and Townson, D. H. (1996). Prolactin-induced regression of the rat corpus luteum: expression of monocyte chemoattractant protein-1 and invasion of macrophages. *Biol Reprod* **54** : 1120-1127.
- Brach, M., Gruss, H., Riedel, D., Asano, Y., DeVos, S. and Herrmann, F. (1992). Effect of anti-inflammatory agents on synthesis of MCP-1 / JE transcripts by human blood monocytes. *Mol Pharmacol* **42** : 63-68.
- Brenner, R. M., Rudolph, L., Matrisian, L. and Slayden, O. D. (1996). Non-human primate models: artificial menstrual cycle, endometrial matrix metalloproteinases and s.c. endometrial grafts. *Hum Reprod* **11** Suppl 2 : 150-164.
- Brooks, J., Holland, P. and Kelly, R. W. (1990). Comparison of antiprogesterin stimulation of uterine prostaglandin synthesis *in vitro*. *Prostaglandins Leukot Essent Fatty Acids* **40** : 191-197.

Brown, Z., Gerritsen, M. E., Carley, W. W., Strieter, R. M., Kunkel, S. L. and Westwick, J. (1994). Chemokine gene expression and secretion by cytokine activated human microvascular endothelial cells - differential regulation of monocyte chemoattractant protein-1 and interleukin-8 in response to interferon- $\gamma$ . *Am J Pathol* **145** : 913-921.

Buckley, C. H. and Fox, H. (1989). Biopsy Pathology of the Endometrium. In (Eds) Gottlieb, L. S., Neville, A. M. and Walker, F. Biopsy Pathology Series. Chapman and Hall Medical, London.

Buhimschi, I., Ali, M., Jain, V., Chwalisz, K. and Garfield, R. E. (1996). Different regulation of nitric oxide in the rat uterus and cervix during pregnancy and labour. *Hum Reprod* **11** : 1755-1766.

Bulmer, J. N., Lunny, D. P. and Hagin, S. V. (1988). Immunohistochemical characterisation of stromal leukocytes in non-pregnant human endometrium. *Am J Reprod Immunol* **17** : 83-90.

Bulmer, J. N., Longfellow, M. and Ritson, A. (1991a). Leukocytes and resident blood cells in endometrium. *Ann N Y Acad Sci* **622** : 57-68.

Bulmer, J. N., Morrison, L., Longfellow, M., Ritson, A. and Pace, D. (1991b). Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies. *Hum Reprod* **6** : 791-798.

Bulmer, J. N. (1995). Immune cells in decidua. In (Eds) Kurpisz, M. and Fernandez, N. Immunology of Human Reproduction. BIO Scientific Publishers Ltd., Oxford : 313-334.

Byers, M., Kuiper, G. G. J. M., Gustafsson, J. A. and Park-Sarge, O. K. (1997). Estrogen receptor- $\beta$  mRNA expression in rat ovary: downregulation by gonadotropins. *Mol Endocrinol* **11** : 172-182.

Cai, J. P., Hudson, S. and Chin, Y. H. (1995). Stimulation of Human T cell transdermal microvascular endothelial cell migration by MCP-1. *J Invest Dermatol* **104** Suppl : 558.

Calder, A. and Embrey, M. P. (1973). Prostaglandins and the unfavourable cervix. *Lancet* **2** : 1322-1333.

Calder, A. A. (1986). Cervical ripening. In (Eds) Bygdeman, M., Berger, G. S. and Keith, L. G. Prostaglandins and their Inhibitors in Clinical Obstetrics and Gynaecology. MTP Press, Lancaster : 145-164.

Cameron, I. T., Baird, D. and Michie, A. F. (1986a). Therapeutic abortion in early pregnancy with antiprogesterone RU486 alone or in combination with prostaglandin analogue (gemeprost). *Contraception* **34** : 459-468.

Cameron, I. T., Kelly, R. W. and Baird, D. T. (1986b). Dysfunctional uterine bleeding: a role for the prostaglandins. *Br J Obstet Gynaecol* **93** : 90-94.

Cameron, I. T., Leask, R., Kelly, R. W. and Baird, D. T. (1987). Endometrial prostaglandins in women with abnormal menstrual bleeding. *Prostaglandins Leukot Med* **29** : 249-257.

Cameron, I. T. (1989). Dysfunctional uterine bleeding. *Bailliere's Clin Obstet Gynaecol* **3** : 315-327.

Cameron, I. T., Davenport, A. P., van Papendorp, C., Barker, P. J., Huskisson, N. S., Gilmour, R. S., Brown, M. J. and Smith, S. K. (1992). Endothelin-like immunoreactivity in human endometrium. *J Reprod Fert* **95** : 623-628.

Cameron, I. T., Plumpton, C., Champeney, R., van Papendorp, C., Ashby, M. J. and Davenport, R. (1993). Identification of endothelin-1, endothelin-2 and endothelin-3 in human endometrium. *J Reprod Fert* **97** : 251-255.

Cameron, S. T., Critchley, H. O. D., Thong, K. J., Buckley, C. H. and Baird, D. T. (1994). Effects of continuous low dose mifepristone (RU486) on the endometrium. *J Reprod Fert* Abstract series 14 : 12.

Cameron, S. T., Critchley, H. O. D., Buckley, C. H., Chard, T., Kelly, R. W. and Baird, D. T. (1996). The effects of post-ovulatory administration of onapristone on the development of a secretory endometrium. *Hum Reprod* **11** : 40-49.

Cameron, S. T., Critchley, H. O. D., Buckley, C. H., Kelly, R. W. and Baird, D. T. (1997). Effect of two antiprogesterones (mifepristone and onapristone) on endometrial factors of importance for implantation. *Fertil Steril* **67** : 1046-1053.

Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S. and Cerami, A. (1986). Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. *Proc Natl Acad Sci USA* **83** : 1670-1674.

Carson, S. A. (1992). A summary of normal female reproductive physiology. In (Eds) Alexander, N. J. and D'Arcangues, C. *Steroid Hormones and Uterine Bleeding*. AAAS Press, Washington.

Casey, M. L., Hemsell, D. L., Johnston, J. M. and MacDonald, P. C. (1980). NAD dependent 15-hydroxyprostaglandin dehydrogenase activity in human endometrium. *Prostaglandins* **19** : 115 -122.

Casey, M. L., Smith, J. W., Hagai, K., Hersh, L. B. and MacDonald, P. C. (1991). Progesterone-regulated cyclic modulation of membrane metalloendopeptidase (enkephalinase). *J Biol Chem* **266** : 23041-23047.

Casey, M. L. and MacDonald, P. C. (1993). Cytokines in the human placenta, fetal membranes, uterine decidua and amniotic fluid. In (Eds) Rice, G. E. and Brennecke, S. P. *Molecular Aspects of Placental and Fetal Membrane Autocoids*. CRC Press, Boca Raton.

Castracane, V. D. and Jordan, V. C. (1975). The effect of oestrogen and progesterone on uterine prostaglandin biosynthesis in the ovariectomised rat. *Biol Reprod* **13** : 587-596.

Catt, K. J. (1996). Hormone Receptors. In (Eds) Hillier, S. G., Kitchner, N. C. and Neilson, J. P. *Scientific Essentials of Reproductive Medicine*. W B Saunders, London : 32-44.

Chambers, W. H., Amoscato, A. A., Smith, M. S., Kenniston, T. W., Herberman, R. B. and Appasamy, P. M. (1995). Prolactin receptor expression by rat NK cells. *Nat Immunol* **14** : 145-156.

Chaouat, G., Menu, F., Clark, D. A., Dy, M., Minkowaki, M. and Wegmann, T. G. (1990). Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. *J Reprod Fertil* **89** : 447-458.



Charnock-Jones, D., Sharkey, A., Fenwick, P. and Smith, S. K. (1994). Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *Reprod Fertil* **101** : 421-426.

Chen, D.-B., Hilsenrath, R., Yang, Z.-M., Le, S.-P., Kim, S.-R., Chuong, C. J., Poindexter III, A. N. and Harper, M. J. K. (1995a). Leukaemia inhibitory factor in human endometrium during the menstrual cycle: cellular origin and action on production of glandular epithelial cell prostaglandin *in vitro*. *Hum Reprod* **10** : 911-918.

Chen, D. B., Yang, Z. M., Hilsenrath, R., Le, S. P. and Harper, M. J. K. (1995b). Stimulation of prostaglandin (PG) F<sub>2α</sub> and PGE<sub>2</sub> release by tumour necrosis factor-α and interleukin-1α in cultured human luteal phase endometrial cells. *Mol Hum Reprod* **1** : 2773-2780.

Cheng, L., Kelly, R. W., Thong, K. J., Hume, R. and Baird, D. T. (1993a). The effect of mifepristone (RU486) on the immunohistochemical distribution of prostaglandin E and its metabolite in decidual and chorionic tissue in early pregnancy. *J Clin Endocrinol Metab* **77** : 873-877.

Cheng, L., Kelly, R. W., Thong, K. J., Hume, R. and Baird, D. T. (1993b). The effects of mifepristone (RU486) on prostaglandin dehydrogenase in decidual and chorionic tissue in early pregnancy. *Hum Reprod* **8** : 705-709.

Chensue, S. W., Warmington, K. S., Ruth, J. H., Sanghi, P. S., Lincoln, P. and Kunkel, S. L. (1996). Role of monocyte chemoattractant protein-1 (MCP-1) in TH1 (mycobacterial) and TH2 (schistosomal) antigen-induced granuloma-formation - relationship to local inflammation, TH cell expression and IL-12 production. *J Immunol* **157** : 4602-4608.

Chilton, B. S. (1985). Influence of prolactin secretion on DNA synthesis and glandular differentiation in rabbit uterine endometrium. In (Eds) MacLoed, R. M., Thorner, M. O. and Scapagnini, U. *Prolactin, Basic and Clinical Correlates*. Liviana Press, Padova : 351-359.

Chrousos, G. P. (1995). The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. *N Engl J Med* **332** : 1351-1362.



- Chuntharapai, A. and Kim, K. J. (1995). Regulation of the expression of IL-8 receptor A/B by IL-8: possible functions of each receptor. *J Immunol* **155** : 2587-2594.
- Chwalisz, K., Benson, M., Scholz, P., Daum, J., Beier, H. M. and Hegele-Hartung, C. (1994). Cervical ripening with the cytokines interleukin-8, interleukin-1 $\beta$  and tumour necrosis factor- $\alpha$  in guinea-pigs. *Hum Reprod* **9** : 2173-2181.
- Chwalisz, K., Shao-Qing, S., Garfield, R. E. and Beier, H. M. (1997). Cervical ripening in guinea-pigs after a local application of nitric oxide. *Hum Reprod* **12** : 2093-2101.
- Clapp, C. and Weiner, R. I. (1992). A specific, high affinity, saturable binding site for the 16-kilodalton fragment of prolactin on capillary endothelial cells. *Endocrinology* **130** : 1380-1386.
- Clapp, C., Martial, J. A., Guzman, R. C., Rentier-Delrue, F. and Weiner, R. I. (1993). The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. *Endocrinology* **133** : 1292-1299.
- Clark, D. A., Wang, S. L., Rogers, P., Vince, G. and Affandi, B. (1996). Endometrial lymphomyeloid cells in abnormal uterine bleeding due to levonorgestrel (Norplant). *Hum Reprod* **11** : 1438-1444.
- Clarke, C. L. and Sutherland, R. L. (1990). Progestin regulation of cellular proliferation. *Endocr Rev* **11** : 266 - 301.
- Clarke, L. A., Edery, M., Loudon, A. S. I., Randall, V. A., Postel-Vinay, M. C., Kelly, P. A. and Jabbour, H. N. (1995). Expression of the prolactin receptor gene during the breeding and non-breeding seasons in red deer (*Cervus elephus*) : evidence for the expression of two forms in the testis. *J Endocrinol* **146** : 313-321
- Clemens, L. W. (1977). Inhibition by progesterone of leukocyte migration *in vitro* and *in vivo*. *Abstracts of the 59th Annual Meeting of the Endocrine Society* : 175 (Abstract No. 237).
- Clevenger, C. V., Russell, D. H., Appasamy, P. M. and Prystowsky, M. B. (1990). Regulation of interleukin 2-driven T-Lymphocyte proliferation by prolactin. *Proc Natl Acad Sci USA* **87** : 6460-6464.

Clevenger, C. V., Sillman, A. L. and Prystowky, M. B. (1990). Interleukin-2 driven nuclear translocation of prolactin in cloned T-lymphocytes. *Endocrinology* **127** : 3151-3159.

Cocchi, F., Devico, A. L., Garzinodemo, A., Arya, S. K., Gallo, R. C. and Lusso, P. (1995). Identification of RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  as the major HIV-suppressive factors produced by CD8(+) T-cells. *Science* **270** : 1811-1815.

Colditz, I. G. (1990). Effect of exogenous prostaglandin E<sub>2</sub> and actinomycin D on plasma leakage induced by neutrophil activating peptide-1/interleukin-8. *Immunol Cell Biol* **68** : 397 - 403.

Cole, O. F., Hiroyuki, S., Sullivan, H. M. F. and Elder, M. G. (1995). Interleukin-1 $\beta$  stimulated prostaglandin synthesis by human decidual cells is independent of protein kinase C. *Prostaglandins* **49** : 69-77.

Coleman, R. A., Smith, W. L. and Narumiya, S. (1994a). VIII. International Union of Pharmacology. Classification of prostanoid receptors: Properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* **46** : 205-225.

Coleman, R. A., Grix, S. P., Head, S. A., Louttit, J. B., Mallett, A. and Sheldrick, R. L. G. (1994b). A novel inhibitory receptor in piglet saphenous vein. *Prostaglandins* **47** : 151-168.

Combadiere, C., Ahuja, S. K., Van Damme, J., Tiffany, H. L., Gao, J. L. and Murphy, P. M. (1995). Monocyte chemoattractant protein-3 is a functional ligand for CC chemokine receptor-1 and receptor-2B. *J Biol Chem* **270** : 29671-29675.

Critchley, H. O. D., Bailey, D. A., Au, C. L., Affandi, B. and Rogers, P. (1993). Immunohistochemical sex steroid receptor distribution in endometrium from long-term subdermal levonorgestrel users and during the normal menstrual cycle. *Hum Reprod* **8** : 1632-1639.

Critchley, H. O. D., Kelly, R. W. and Kooy, J. (1994). Perivascular expression of chemokine interleukin-8 in human endometrium. *Hum Reprod* **9** : 1406-1409.

Critchley, H. O. D., Kelly, R. W., Lea, R. G., Drudy, T. A., Jones, R. L. and Baird, D. T. (1996). Sex steroid regulation of leucocyte traffic in human decidua. *Hum Reprod* **11** : 2257-2262.

Critchley, H. O. D., Wang, H., Kelly, R. W., Gebbie, A. E. and Glasier, A. F. (1998a). Progesterin receptor isoforms and prostaglandin dehydrogenase in the endometrium of women using a levonorgestrel-releasing intra-uterine system. *Hum Reprod* : **In Press**.

Critchley, H. O. D., Wang, H., Jones, R. L., Kelly, R. W., Drudy, T., Gebbie, A. E., Buckley, C. H., McNeilly, A. S. and Glasier, A. F. (1998b). Morphological and functional features of endometrial decidualisation following long term intra uterine levonorgestrel delivery. *Hum Reprod* : **In Press**.

Critchley, H. O.D., Jones, R. L., Lea, R. G., Drudy, T. A., Kelly, R. W., Williams, A. R. and Baird, D. T. (1998c). The role of progesterone in expression of inflammatory mediators in human endometrium. *J Clin Endocrinol Metab* : **Submitted**.

Csapo, A. I., Pulkkinen, M. O. and Wiest, W. G. (1973). Effects of luteectomy and progesterone replacement therapy in early pregnant patients. *Am J Obstet Gynecol* **115** : 759-765.

Da Silver, J. A. and Spector, T. D. (1992). The role of pregnancy in the course and aetiology of rheumatoid arthritis. *Clin Rheumatol* **11** : 189.

Dallenbach-Hellweg, G. (1987). Histopathology of the endometrium. Springer-Verlag, New York : 190-198.

Daly, D. C., Maslar, I. A. and Riddick, D. H. (1983). Prolactin production during *in vitro* decidualisation of proliferative endometrium. *Am J Obstet Gynecol* **145** : 672-678.

Dardenne, M., Do Carmo, M., De Moraes, L., Kelly, P. A. and Gagnerault, M. (1994). Prolactin receptor expression in human haematopoietic tissues analyzed by flow cytofluorometry. *Endocrinology* **134** : 2108-2114.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. and de Vries, J. E. (1991). Interleukin-10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* **174** : 1209-1220.

Deb, S., Ou, J., Park-Sarge, O. K. and Gibori, G. (1997). Estrogen receptor- $\beta$  is expressed in the rat decidual tissue and mediates estradiol action. *Biol Reprod* **56** Suppl 1 : 221.

Dennes, W. J. B., Slater, D. M. and Bennett, P. R. (1997). Nitric oxide synthase mRNA expression in human fetal membranes: a possible role in parturition. *Biochem Biophys Res Commun* **233** : 276-278.

DeWitt, D. L. (1991). Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochim Biophys Acta* **1083** : 121-134.

Dietl, J., Ruck, P., Marzusch, K., Horny, H.-P., Kaiserling, E. and Handgretinger, R. (1992). Uterine granular lymphocytes are activated natural killer cells. *Immunol Today* **13** : 236.

Dijane, J., Clauser, H. and Kelly, P. A. (1979). Rapid down regulation of prolactin receptors in mammary gland and liver. *Biochem Biophys Res Commun* **90** : 1371-1378.

DiMattia, G. E., Gellerson, B., Duckworth, M. L. and Friesen, H. G. (1990). Human prolactin gene expression. The use of an alternative noncoding exon in decidua and the IM-9-P3 lymphoblast cell line. *J Biol Chem* **265** : 16412-16421.

Dineen, J. K. and Kelly, J. D. (1972). The suppression of rejection of *Nippostrongylus brasiliensis* in lactating rats : the nature of the immunological defect. *Immunol* **22** : 1-13.

Downie, J., Poyser, N. L. and Wunderlich, M. (1974). Levels of prostaglandins in human endometrium throughout the menstrual cycle. *J Physiol* **236** : 465-472.

Draca, S. (1995). Prolactin as an immunoreactive agent - review. *Immunol Cell Biol* **73** : 481-483.

Drake, B. L. and Head, J. R. (1989). Murine trophoblast can be killed by lymphokine-activated killer cells. *J Immunol* **143** : 9-14.

Dudley, D. J., Trautman, M. S. and Mitchell, M. D. (1993). Inflammatory mediators regulate IL-8 production by cultured gestational tissues : Evidence for a cytokine network at the chorio-decidual interface. *J Clin Endocrinol Metab* **76** : 404-410.

Edwards, R. G. (1995). Physiological and molecular aspects of human implantation. *Hum Reprod* **10** Suppl 2 : 1-13.

Edwards, R. G. and Surani, M. A. H. (1978). The primate blastocyst and its environment. *Uppsala J Med Sci* **22** : 816-818.

- Edwards, S. W. and Hallett, M. B. (1997). Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. *Immunol Today* **18** : 320-324.
- Eisenberg, S. P., Evans, R. J., Arend, W. P., Verderber, E., Brewer, M. T., Hannum, C. H. and Thompson, R. C. (1990). Primary structure and functional expression from complementary-DNA of a human interleukin-1 receptor antagonist. *Nature* **343** : 341-346.
- El Maradny, E., Kanayama, N., Halim, A., Maehara, K., Suminoto, K. and Terao, T. (1996). Biochemical changes in the cervical tissue of rabbit induced by interleukin-8, interleukin-1 $\beta$ , dehydroepiandrosterone sulphate and prostaglandin E<sub>2</sub>: a comparative study. *Hum Reprod* **11** : 1099-1104.
- Elgberg, G., Kelly, P. A., Dijane, J., Binder, L. and Gertler, A. (1990). Mitogenic and binding properties of monoclonal antibodies to the prolactin receptor in Nb2 rat lymphoma cells. Selective enhancement by anti-mouse IgG. *J Biol Chem* **265** : 14770-14776.
- Ellis, S. A., Palmer, M. S. and McMichael, A. J. (1990). Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA Class I molecule. *J Immunol* **144** : 731-735.
- Fakih, H., Baggett, B., Holtz, G., Tsang, K. Y., Lee, J. C. and Williamson, H. O. (1987). Interleukin-1 - a possible role in the infertility associated with endometriosis. *Fertil Steril* **47** : 213-217.
- Feliciani, C., Theoharides, T. C., Mammarella, S., Letourneau, R., Reale, M., Barbacane, R., Amerio, P. and Conti, P. (1995). Activation of mast cells by MCP-1. *J Invest Dermatol* **104** : 685.
- Feng, Y., Broder, C. C., Kennedy, P. E. and Berger, E. A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven transmembrane, G protein coupled receptor. *Science* **272** : 872-877.
- Findlay, J. K. (1996). Future directions for research on endometrial bleeding. *Hum Reprod* **11** Suppl 2 : 179-183.
- Finn, C. A. and Pope, M. D. (1984). Vascular and cellular changes in the decidualised endometrium of the ovariectomised mouse following cessation of hormone treatment: a possible model for menstruation. *J Endocrinol* **100** : 295-300.



- Finn, C. A. (1986). Implantation, menstruation and inflammation. *Biol Reviews* **61** : 313-328.
- Finn, C. A. (1994). The adaptive significance of menstruation. *Hum Reprod* **9** : 1202-1207.
- Fiorentino, D. F., Bond, M. W. and Mosmann, T. R. (1989). Two types of mouse T helper cell. IV. TH2 clones secrete a factor that inhibits cytokine production by TH1 clones. *J Exp Med* **170** : 2081.
- Foster, S. J., M, A. D., M, S. J. and E, C. (1989). Acute inflammatory effects of a monocyte-derived neutrophil activating peptide in rabbit skin. *Immunology* **67** : 181-183.
- Frenette, P. S. and Wagner, D. D. (1996). Molecular medicine; Adhesion molecules - part 1. *N Engl J Med* **34** : 1526-1529.
- Freter, R. R., Alberta, J. A., Lam, K. K. and Stiles, C. D. (1995). A new platelet-derived growth factor-regulated genomic element which binds a serine threonine phosphoprotein mediates induction of the slow immediate early gene MCP-1. *Mol Cell Biol* **15** : 315-325.
- Fried, G. and Samuelson, U. (1991). Endothelin and neuropeptide Y are vasoconstrictors in human blood vessels. *Am J Obstet Gynecol* **164** : 1330-1336.
- Futaki, N., Takahashi, S., Yokoyama, M., Arai, I., and Higuchi, S. (1994). NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. *Prostaglandins* **47** : 55-59.
- Gabbiani, G. (1992). The biology of the myofibroblast. *Kidney Int* **41** : 530-532.
- Gao, J.-L., Kuhns, D. B., Tiffany, H. L., McDermott, D., Li, X., Francke, U. and Murphy, P. M. (1993). Structure and functional expression of the human macrophage inflammatory protein 1 $\alpha$  / RANTES receptor. *J Exp Med* **177** : 1421-1427.
- Gao, J.-L., Wynn, T. A., Chang, Y., Lee, E. J., Broxmeyer, H. E., Cooper, S., Tiffany, H. L., Westphal, H., Kwon-Chung, J. and Murphy, P. M. (1997). Impaired host defence, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J Exp Med* **185** : 1959-

1968.

Garcia, E., Bouchard, P., DeBrux, J., Berdah, J., Frydman, R., Schaison, G., Milgrom, E. and Perrot-Applanat, M. (1988). Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating. *J Clin Endocrinol Metab* **67** : 80-87.

Gauldie, J., Richards, C., Harnish, D., Lansdorp, P. and Baumann, H. (1987). Interferon- $\beta$ /B cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte stimulating factor and regulates the major acute phase protein response in liver cells. *Proc Natl Acad Sci USA* **84** : 7251-7255.

Gellerson, B., Kempf, R., Telgmann, R. and DiMattia, G. E. (1994). Nonpituitary human prolactin gene transcription is independent of Pit-1 and differentially controlled in lymphocytes and in endometrial stroma. *Mol Endocrinol* **8** : 356-373.

Gemzell-Danielsson, K., Svalander, P., Swahn, M. L., Johannisson, E. and Bygdeman, M. (1994). Effects of a single post-ovulatory dose of RU486 on endometrial maturation in the implantation phase. *Hum Reprod* **9** : 2398-2404.

Gemzell-Danielsson, K., Swahn, M. L. and Bygdeman, M. (1997a). The effect of various doses of mifepristone on endometrial leukaemia inhibitory factor expression in the midluteal phase - an immunohistochemical study. *Hum Reprod* **12** : 1293-1297.

Gemzell-Danielsson, K., Swahn, M. L., Westland, P. Johannisson, E., Seppala, M. and Bygdeman, M. (1997b). Effect of low daily doses of mifepristone on ovarian function and endometrial development. *Hum Reprod* **12** : 124-151.

Gibb, W. and Sun, M. (1996). Localisation of prostaglandin-H synthase type 2 (PGHS-2) protein and mRNA in term human fetal membranes and decidua. *J Endocrinol* **150** : 497-503.

Gierse, J. K., Hauser, S. D., Creely, D. P., Koboldt, C., Rangwala, S. H., Isakson, P. C. and Seibert, K. (1995). Expression and selective inhibition of the constitutive and inducible forms of cyclooxygenase. *Biochem J* **305** : 479-484.

Godiska, R., Chantry, D., Raport, C. J., Schweickart, V. L., Trong, H. L. and Gray, P. W. (1997). Monocyte chemotactic protein-4: tissue specific expression and signalling through CC chemokine receptor-2. *J Leuk Biol* **61** : 353-360.



- Goodger, A. M. and Rogers, P. A. W. (1994). Endometrial endothelial cell proliferation during the menstrual cycle. *Hum Reprod* **9** : 399-405.
- Goppelt-Streube, M. (1995). Regulation of prostaglandin endoperoxide synthase (cyclo-oxygenase) isoenzyme expression. *Prostaglandins Leukot Essent Fatty Acids* **52** : 213-222.
- Gu, L., Rutledge, B., Fiorillo, J., Ernst, C., Grewal, I., Flavell, K., Gladue, R. and Rollins, B. (1997). *In vivo* properties of monocyte chemoattractant protein-1. *J Leuk Biol* **62** : 577-580.
- Gu, Y., Soares, M. J., Srivastava, R. K. and Gibor, G. (1994). Expression of decidual prolactin-related protein in the rat decidua. *Endocrinology* **135** : 1422-1427.
- Guilbert, L. J. (1996). There is a bias against type 1 (inflammatory) cytokine expression and function in pregnancy. *J Reprod Immunol* **32**
- Gumperz, J. E. and Parham, P. (1995). The enigma of the natural killer cell. *Nature* **378** : 245-248.
- Gura, T. (1996). Chemokines take center stage in inflammatory ills. *Science* **272** : 954-956.
- Gustafsson, J. A. (1997). Estrogen receptor  $\beta$  - getting in on the action? *Nat Med* **3** : 493-494.
- Hadley, T. J., Lu, Z.-h., Wasniowska, K., Martin, A. W., Peiper, S. C., Hesselgesser, J. and Horuk, R. (1994). Postcapillary venule endothelial cells in kidney express a multispecific chemokine receptor that is structurally and functionally identical to the erythroid isoform which is the Duffy blood group antigen. *J Clin Invest* **94** : 985-991.
- Hadley, T. J. and Peiper, S. C. (1997). From malaria to chemokine receptor: the emerging physiologic role of the duffy blood group antigen. *Blood* **89** : 3077-3091.
- Haines and Taylor (1987). Obstetrical and Gynaecological Pathology. In (Eds) Fox, H. *Obstetrical and Gynaecological Pathology*. Churchill Livingstone, London, Edinburgh, Melbourne, New York.

- Haller, H., Radillo, O., Rukavina, D., Tedesco, F., Candussi, G., Petrovic, O. and Randic, L. (1993). An immunohistochemical study of leukocytes in human endometrium, first and third trimester basal decidua. *J Reprod Immunol* **23** : 41-49.
- Halme, J., Becker, S., Hammond, M. G., Raj, M. H. G. and Raj, S. (1983). Increased activation of pelvic macrophages in infertile women with mild endometriosis. *Am J Obstet Gynecol* **145** : 333-337.
- Halme, J., Becker, S. and Haskill, S. (1987). Altered maturation and function of peritoneal macrophages: possible role in pathogenesis of endometriosis. *Am J Obstet Gynecol* **156** : 783-789.
- Halme, J. (1989). Release of tumor necrosis factor-alpha by human peritoneal-macrophages *in vivo* and *in vitro*. *Am J Obstet Gynaecol* **161** : 1718-1725.
- Hampton, A. L. and Salamonsen, L. A. (1994). Endometrial expression of messenger ribonucleic acid encoding matrix metalloproteases and their tissue inhibitors coincides with menstruation. *J Endocrinol* **141** : R1-R3.
- Han, S. W., Lei, Z. M. and Rao, Ch. V. (1996). Up-regulation of cyclooxygenase-2 gene expression by chorionic gonadotropin during the differentiation of human endometrial stroma into decidua. *Endocrinology* **137** : 1719-1797.
- Handwerger, S. and Brar, A. (1992). Placental lactogen, placental growth hormone and decidual prolactin. *Semin Reprod Endocrinol* **10** : 106-115.
- Harada, A., Mukaida, N. and Matsushima, K. (1996). Interleukin-8 as a novel target for intervention therapy in acute inflammatory diseases. *Mol Med Today* **2** : 482-489.
- Hartman, D., Holaday, J. and Bernton, E. (1989). Inhibition of lymphocyte proliferation by antibodies to prolactin. *FASEB J* **3** : 2194-2202.
- Hartner, A., Goppelte-Struebe, M., Hocke, G. M. and Sterzel, R. B. (1997). Differential regulation of chemokines by leukaemia inhibitory factor, interleukin-6 and oncostatin M. *Kidney Int* **51** : 1754-1760.

Haskill, S., Martin, G., Vanle, L., Morris, J., Peace, A., Bigler, C. F., Jaffe, G. J., Hammerberg, C., Spron, S. A., Fong, S., Arend, W. P. and Ralph, P. (1991). cDNA cloning of an intracellular form of the human interleukin-1 receptor antagonist associated with epithelium. *Proc Natl Acad Sci USA* **88** : 3682-3685.

Haynes, M. K. and Smith, J. B. (1997). Can Th1-like immune responses explain the immunopathology of recurrent spontaneous miscarriage? *J Reprod Immunol* **35** : 65-71

Healy, D. L. (1985). Chronic polyhydramnios is a syndrome with a lactogen receptor defect in the chorion laeve. *Br J Obstet Gynaecol* **92** : 461-467.

Herrmann, W. I., Schindler, A. M., Wyss, R. and Bischof, P. (1985). Effects of the antiprogesterone RU486 in early pregnancy and during the menstrual cycle. In (Eds) Segal, S. J. and Baulieu, E. *The antiprogesterone steroid RU486 and human fertility control*. Plenum Press, New York : 179-198.

Hilkens, C. M. U., Vermeulen, H., Joost van Neerven, R. J., Snijdwint, F. G. M., Wierenga, E. A. and Kapsenberg, M. L. (1995). Differential modulation of T helper type 1 (Th1) and T helper 2 (Th2) cytokine secretion by prostaglandin E<sub>2</sub> critically depends on interleukin-2. *Eur J Immunol* **25** : 59-63.

Hill, J. A. (1992). Cytokines considered critical in pregnancy. *Am J Reprod Immunol* **28** : 123-126.

Hill, J. A., Polgar, K. and Anderson, D. J. (1995). T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. *JAMA* **273** : 1933.

Hla, T. and Neilson, K. (1992). Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci USA* **89** : 7384-7388.

Hornung, D., Ryan, I. P., Chao, V. A., Vigne, J.-L., Schriock, E. D. and Taylor, R. N. (1997). Immunolocalisation and regulation of the chemokine RANTES in human endometrial and endometriosis tissues and cells. *J Clin Endocrinol Metab* **82** : 1621-1628.

Horuk, R. (1994). The interleukin-8-receptor family: from chemokines to malaria. *Immunol Today* **15** : 169-174.

Houdebine, L. M., Dijane, J., Dusanter-Fourt, I., Martel, P., Kelly, P. A., Devinoy,

- E. and Servely, J. L. (1985). Hormone action controlling mammary gland activity. *J Dairy Sci* **68** : 489-500.
- Huang, M., Stolina, M., Sharma, S., Mao, J. T., Zhu, L., Miller, P. W., Wollman, J., Herschman, H. and Dubinett, S.M. (1998). Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: upregulation of interleukin-10 and down-regulation of interleukin-12 production. *Cancer Res* **58** : 1208-1216.
- Hunt, J. S., Miller, L., Vassmer, D. and Croy, B. A. (1997). Expression of the inducible nitric oxide synthase gene in mouse uterine leukocytes and potential relationships with uterine function during pregnancy. *Biol Reprod* **57** : 827-836.
- Illingworth, P. J., Reddi, K., Smith, K. and Baird, D. T. (1990). Pharmacologic "rescue" of the corpus luteum results in increased inhibin production. *Clin Endocrinol* **33** : 323-332.
- Inoue, T., Kanzaki, H., Imai, K., Narukawa, S., Katsuragawa, H., Watanabe, H., Hirano, T. and Mori, T. (1996). Progesterone stimulates the induction of human endometrial CD56<sup>+</sup> lymphocytes in an *in vitro* culture system. *J Clin Endocrinol Metab* **81** : 1502-1507.
- Ishihara, O., Khan, H., Sullivan, M. H. F. and Elder, M. G. (1992). IL-1 $\beta$  stimulates decidual stromal cell cyclooxygenase enzyme and prostaglandin production. *Prostaglandins* **44** : 43-52.
- Ishihara, O., Matsuoka, K., Kinoshita, K., Sullivan, M. H. F. and Elder, M. G. (1995). Interleukin-1 $\beta$ -Stimulated PGE<sub>2</sub> Production from Early First Trimester Human Decidual cells is Inhibited by Dexamethasone and Progesterone. *Prostaglandins* **49** : 15-26.
- Ito, A., Imada, K., Sato, T., Kubo, T., Matsushima, K. and Mori, Y. (1994). Suppression of interleukin 8 production by progesterone in rabbit uterine cervix. *Biochem J* **301** : 183-186.
- Jabbour, H. N., Critchley, H. O. D. and Boddy, S. (1997). Expression of functional prolactin receptors in non-pregnant human endometrium: Jak2, Stat1 and Stat5 proteins are phosphorylated following stimulation with prolactin. *J Clin Endocrinol Metab* : **In Press**

- Jacobs, A. L., Hwang, D., Julian, J. and Carson, D. D. (1994). Regulated expression of prostaglandin endoperoxide synthase-2 by uterine stroma. *Endocrinology* **135** : 1807-1815.
- Jeziorska, M., Salamonsen, L. A. and Woolley, D. E. (1995). Mast cell and eosinophil distribution and activation in human endometrium throughout the menstrual cycle. *Biol Reprod* **53** : 312-320.
- Jiang, Y., Valente, A. J., Williamson, M. J., Zhang, L. and Graves, D. T. (1990). Post-translational modification of a monocyte-specific chemoattractant synthesised by glioma, osteosarcoma, and vascular smooth muscle cells. *J Biol Chem* **265** : 18318-18321.
- Jikihara, H., Kessler, C. A., Cedars, M. I. and Brar, A. K. (1996). Up regulation of the human prolactin receptor in the endometrium. *Endocrine* **5** : 157-162.
- Johannisson, E., Parker, R. A., Landgren, B. M. and Diczfalusy, E. (1982). Morphometric analysis of the human endometrium in relation to peripheral hormone levels. *Fertil Steril* **38** : 564-571.
- John, M., Au, B.T., Jose, P. J., Lim, S., Saunders, M., Barnes, P. J., Mitchell, J. A., Belvisi, M. G. and Chung, K. T. (1998) Expression and release of interleukin-8 by human airway smooth muscle cells: inhibition by Th-2 cytokines and corticosteroids. *Am J Respir Cell Mol Biol* **18** : 84-90
- Jones, R. L., Kelly, R. W. and Critchley, H. O. D. (1997). Chemokine expression in human endometrium coincides with leukocyte accumulation. *Hum Reprod* **12** : 1300-1306.
- Jones, R. L., Critchley, H. O. D., Brooks, J., Jabbour, H. N. and McNeilly, A. S. (1998). Spatial and temporal expression of prolactin receptor in human endometrium. *J Clin Endocrinol Metab* **83** : 258-262.
- Jonjic, N., Peri, G., Bernasconi, S., Sciacca, F. L., Collotta, F., Pelicci, P., Lanfrancane, L. and Mantovani, A. (1992). Expression of adhesion molecules and chemotactic cytokines in cultured human mesothelial cells. *J Exp Med* **176** : 1165-1174.
- Jun, S. S., Chen, Z., Sherman, T. S., Margraf, L. R. and Shaul, P. W. (1996). Estrogen up-regulates cyclooxygenase gene expression in fetal pulmonary artery



endothelial cells. *Pediatr Res* **39** : 164.

Kaiserman-Abramof, I. R. and Padkula, H. A. (1989). Angiogenesis in the postovulatory primate endometrium: the coiled arteriolar system. *Anat Rec* **224** : 479-489.

Kamat, B. R. and Isaacson, P. G. (1987). The immunocytochemical distribution of leukocytic subpopulations in human endometrium. *Am J Pathol* **127** : 66-73.

Karpus, W. J. and Kennedy, K. J. (1997a). MIP-1 $\alpha$  and MCP-1 differentially regulate acute and relapsing autoimmune encephalomyelitis as well as Th1/Th2 lymphocyte differentiation. *J Leuk Biol* **62** : 681-687.

Karpus, W. J., Lukacs, N. W., Kennedy, K. J., Smith, W. S., Hurst, S. D. and Barrett, T. A. (1997b). Differential CC chemokine-induced enhancement of T helper cell cytokine production. *J Immunol* **158**

Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H. and Chambon, P. (1990). Two distinct estrogen-regulated promoters generate transcripts encoding the 2 functionally different human progesterone receptor form-A and form-B. *EMBO J* **9** : 1603-1614.

Kelly, J. D. and Dineen, J. K. (1973). The suppression of rejection of *Nippostrongylus brasiliensis* in Lewis strain rats treated with ovine prolactin. *Immunol* **24** : 551-558.

Kelly, R. W., Healy, D. L., Cameron, M. J., Cameron, I. T. and Baird, D. T. (1986). The stimulation of prostaglandin production by two antiprogestosterone steroids in human endometrial cells. *J Clin Endocrinol Metab* **62** : 1116-1123.

Kelly, R. W. and Smith, S. K. (1987). Glucocorticoids do not share with progesterone the potent inhibitory action of prostaglandin synthesis in human proliferative phase endometrium. *Prostaglandins* **33** : 919-929. Kelly, R. W. (1994). Pregnancy maintenance and parturition: the role of prostaglandin in manipulating the immune and inflammatory response. *Endocr Rev* **15** : 684-706.

Kelly, R. W., Leask, R. and Calder, A. A. (1992). Choriodecidual production of IL-8 and mechanism of parturition. *Lancet* **339** : 776-777.

Kelly, R. W., Illingworth, P., Baldie, G., Leask, R., Brouwer, S. and Calder, A. A.

(1994). Progesterone control of interleukin-8 production in endometrium and chorio-decidual cells underlines the role of the neutrophil in menstruation and parturition. *Hum Reprod* **9** : 253-258.

Kelly, R. W. (1996). Inflammatory mediators and parturition. *Rev Reprod* **1** : 89-96.

Kelly, R. W. and Critchley, H. O. D. (1997a). A T-helper-2 bias in decidua: the prostaglandin contribution of the macrophage and trophoblast. *J Reprod Immunol* **33** : 181-187.

Kelly, R. W., Carr, G. G. and Riley, S. C. (1997b). The inhibition of synthesis of a  $\beta$ -chemokine, monocyte chemotactic protein-1 (MCP-1), by progesterone. *Biochem Biophys Res Commun* **239** : 557-561.

Kelner, G. S., Kennedy, J., Bacon, K. B., Kleyensteuber, S., Largaespada, D. A., Jenkins, N. A., Copeland, N. G., Bazan, J. F., Moore, K. W., Schall, T. J. and Zlotnik, A. (1994). Lymphotoxin : a cytokine that represents a new class of chemokine. *Science* **266** : 1395-1399.

Kennedy, T. G. and Armstrong, D. T. (1981). The role of prostaglandins in endometrial vascular changes at implantation. In (Eds) Glasser, S. R. and Bullock, D. W. *Cellular and Molecular Aspects of Implantation*. Plenum Press, New York and London : 349-363.

Kennedy, T. G., Martel, D. and Psychoyos, A. (1983a). Endometrial prostaglandin E<sub>2</sub> binding: characterisation in rats sensitised for the decidual cell reaction and changes during pseudopregnancy. *Biol Reprod* **29** : 556-564.

Kennedy, T. G., Martel, D. and Psychoyos, A. (1983b). Endometrial prostaglandin E<sub>2</sub> binding during the estrus cycle and its hormonal control in ovariectomized rats. *Biol Reprod* **29** : 565-573.

Kennedy, T. G. (1986). Prostaglandins and uterine sensitisation for the decidual cell reaction. *Ann N Y Acad Sci* **476** : 43-48.

Kimber, S. J. (1994). Carbohydrates and implantation of the mammalian embryo. In (Eds) Glasser, S. R., Mulholland, J. and Psychoyos, A. *Endocrinology of embryo-endometrium interactions*. Plenary Press, New York : 279.



King, A., Balendran, N., Wooding, P., Carter, N. P. and Loke, Y. W. (1991). CD3-Leukocytes Present in the Human Uterus During Early Pregnancy: Phenotypic and Morphologic Characterisation of the CD56++ Population. *Dev Immunol* **1** : 169-190.

King, A., Birkby, C. and Loke, Y. W. (1989). Early human decidual cells exhibit NK activity against K562 cell line but not against first trimester trophoblast. *Cell Immunol* **118** : 337-344.

King, A. and Loke, Y. W. (1990). Uterine large granular lymphocytes: a possible role in embryonic implantation? *Am J Obstet Gynecol* **162** : 308-310.

King, A. and Loke, Y. W. (1991). On the nature and function of human uterine granular lymphocytes. *Immunol Today* **12** : 432-435.

King, A., Wheeler, R., Carter, N. P., Francis, D. P. and Loke, Y. W. (1992). The response of human decidual leukocytes to IL-2. *Cell Immunol* **140** : 406-421.

King, A., Wooding, P., Gardner, L. and Loke, Y. W. (1993). Expression of perforin, granzyme A and TIA-1 by human uterine CD56<sup>+</sup> NK cells implies they are activated and capable of effector functions. *Hum Reprod* **8** : 2061-2067.

King, A., Boocock, C., Sharkey, A. M., Gardner, L., Beretta, A., Siccardi, A. G. and Loke, Y. W. (1996a). Evidence for the expression of HLA-C class I mRNA and protein by human first trimester trophoblast. *J Immunol* **156** : 2068-2076.

King, A., Gardner, L. and Loke, Y. W. (1996b). Evaluation of oestrogen and progesterone receptor expression in uterine mucosal lymphocytes. *Hum Reprod* **11** : 1079-1082.

Klentzeris, L. D., Bulmer, J. N., Warren, A., Morrison, L., Li, T.-C. and Cooke, I. D. (1992). Endometrial lymphoid tissue in the timed endometrial biopsy: Morphometric and immunohistochemical aspects. *Am J Obstet Gynecol* **167** : 666-674.

Klentzeris, L. D., Bulmer, J. N., Tredosiewicz, L. K., Morrison, L. and Cooke, I. D. (1993). Beta-1 integrin cell adhesion molecules in the endometrium of fertile and infertile women. *Hum Reprod* **8** : 1223-1330.

- Klentzeris, L. D. (1997). Adhesion molecules in reproduction. *Br J Obstet Gynaecol* **104** : 401-409.
- Koay, E. S. C., Bryant-Greenwood, G. D., Yamamoto, S. Y. and Greenwood, F. C. (1986). The human fetal membranes : a target tissue for relaxin. *J Clin Endocrinol Metab* **62** : 513-521.
- Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G. and Strieter, R. M. (1992). Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* **258** : 1798-1801.
- Kohnen, G., Irvine, G., Campbell, S., Louis, E., Maclachlan, F., Young, A., Titterton, D. M. and Cameron, I. T. (1997). Expression of endothelin receptors in human decidua. *J Pathol* **182** : A12.
- Koopman, W. and Krangel, M. S. (1997). Identification of a glycosaminoglycan-binding site in chemokine macrophage inflammatory protein-1 $\alpha$ . *J Biol Chem* **272** : 10103-10109.
- Kovacs, E. J., DonCarlos, L. L. and Frazier-Jensen, M. R. (1993). Estrogen inhibits JE/MCP-1 expression in macrophages. *J Immunol* **150** : A84.
- Kovacs, E. J., Faunce, D. E., Ramerquinn, D. S., Mott, F. J., Dy, P. W. W. and Frazierjessen, M. R. (1996). Estrogen regulation of JE/MCP-1 mRNA expression in fibroblasts. *J Leuk Biol* **59** : 562-568.
- Kovats, S. et al. (1990). A class I antigen, HLA-G, expressed in human trophoblasts. *Science* **248** : 220-223.
- Kraan, T. C. T. M. V., Boeijs, L. C. M., Smeenk, R. J. T., Wijdenes, J. and Aarden, L. A. (1995). Prostaglandin-E<sub>2</sub> Is a potent inhibitor of human interleukin-12 production. *J Exp Med* **181** : 775-779.
- Krishnan, L., Gilbert, L. J., Russell, A. S., Wegman, T. G., Mosmann, T. R. and Belosevic, M. (1996). Pregnancy impairs resistance of C57BL/6 mice to *Leishmania major* infection and causes decreased antigen-specific IFN- $\gamma$  responses and increased production of T helper 2 cytokines. *J Immunol* **156** : 644-652.
- Kubba, A. and Guillebaud, J. (1993). Combined oral contraceptives: acceptability and effective use. *Br Med Bull* **49** : 140-157.

Kubjuba, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W. and Herschman, H. R. (1991). TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase / cyclooxygenase homologue. *J Biol Chem* **266** : 12866-12872.

Kubjuba, D. A. and Herschman, H. R. (1992). Dexamethasone inhibits mitogen induction of the TIS10 prostaglandin synthase / cyclooxygenase gene. *J Biol Chem* **267** : 7991-7994.

Kuiper, G. G. J. M., Enmark, E., Peltö-Hikko, M., Nilsson, S. and Gustafsson, J. A. (1996). Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* **93** : 5925-5930.

Kunkel, S. (1988). The importance of arachidonate metabolism by immune and nonimmune cells. *Lab Invest* **58** : 119-121.

Kunkel, S. L. (1996). Th1- and Th2-type cytokines regulate chemokine expression. *Biol Signals* **5** : 197-202.

Kurt-Jones, E. A., Hamberg, S., Ohara, J., Paul, W. E. and Abbas, A. K. (1987). Heterogeneity of helper / inducer T lymphocytes, I: lymphokine production and lymphokine responses. *J Exp Med* **166** : 1774-1787.

Lachapelle, M.-H., Miron, P., Hemmings, R. and Roy, D. C. (1996). Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion: Altered profile and pregnancy outcome. *J Immunol* **156** : 4027-4034.

Laird, S. M., Li, T. C. and Bolton, A. E. (1993). The production of placental protein 14 and interleukin 6 by human endometrial cells in culture. *Hum Reprod* **8** : 793-798.

Lanier, L. L., Le, A. M., Civin, C. I., Loken, M. R. and Phillips, J. H. (1986). The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol* **136** : 4480-4486.

Lanier, L. L., Testi, R., Binal, J. and Phillipps, J. H. (1989). Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J Exp Med* **169** : 2233-2238.

- Lanier, L. L. and Philips, J. H. (1996). Inhibitory MHC class I receptors on NK cells and T cells. *Immunol Today* **17** : 86-91.
- Larsen, C. G., Andeson, A. O., Appella, E., Oppenheim, J. J. and Matsushima, K. (1989a). The neutrophil-activating peptide (NAP-1) is also chemotactic for T lymphocytes. *Science* **243** : 1464-1466.
- Larsen, C. G., Zachariae, C. O., Oppenheim, J. J. and Matsushima, K. (1989b). Production of monocyte chemotactic and activating factor (MCAF) by human dermal fibroblasts in response to interleukin-1 or tumour necrosis factor. *Biochem Biophys Res Commun* **160** : 1403.
- Lau, I. F., Saksena, S. K. and Chang, M. C. (1973). Pregnancy blockage by indomethacin, an inhibitor of prostaglandin synthesis: its reversal by prostaglandins and progesterone in mice. *Prostaglandins* **4** : 795-803.
- Lea, R. G. and Clark, D. A. (1991). Macrophages and migratory cells in endometrium relevant to implantation. *Bailliere's Clin Obstet Gynaecol* **5** : 25-59.
- Lea, R. G., Tulppala, M. and Critchley, H. O. D. (1997). Deficient syncytiotrophoblast tumour necrosis factor- $\alpha$  characterises failing first trimester pregnancies in a subgroup of recurrent miscarriage patients. *Hum Reprod* **12** : 1313-1320.
- Leite de Moraes, M. C., Touraine, P., Gagnerault, M. C., Savino, W., Kelly, P. A. and Dardenne, M. (1995). Prolactin receptors and the immune system. *Annales d'Endocrinologie* **56** : 567-570.
- Leslie, C. A. and Dubey, D. P. (1994). Increased PGE<sub>2</sub> from human monocytes isolated in the luteal phase of the menstrual cycle. Implications for immunity? *Prostaglandins* **47** : 41-54.
- Lessey, B. A., Killam, A. P., Metzger, D. A., Haney, A. F., Greene, G. L. and McCarty, K. S. (1988). Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J Clin Endocrinol Metab* **67** : 334-340.
- Lessey, B. A., Castelbaum, A. J. and Buck, C. A. (1994). Further characterisation of endometrial integrins during the menstrual cycle and in pregnancy. *Fertil Steril* **62** : 497-506.



- Lessey, B. A., Yeh, I., Castelbaum, A. J., Fritz, M. A., Ilesanmi, A. O., Korzeniowski, P., Sun, J. and Chwalisz, K. (1996). Endometrial progesterone receptors and markers of uterine receptivity in the window of implantation. *Fertil Steril* **65** : 477-483.
- Lim, H. and Dey, S. K. (1997a). Prostaglandin E<sub>2</sub> receptor subtype EP2 gene expression in the mouse uterus coincides with differentiation of the luminal epithelium for implantation. *Endocrinology* **138** : 4599-4606.
- Lim, H., Paria, B. C., Das, S. K., Dinchuk, J. E., Langenbach, R., Trzaskos, J. M. and Dey, S. K. (1997b). Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91** : 197-208.
- Lim, K. J., Odukoya, O. A., Ajjan, R. A., Li, T. C., Weetman, A. P., Cooke, I.D. (1998). Profile of cytokine mRNA expression in peri-implantation human endometrium. *Mol Hum Reprod* **4** : 77-81.
- Lin, H., Mosman, T. R., Guilbert, L., Tuntipopipot, S. and Wegman, T. G. (1993). Synthesis of T-helper 2-type cytokines at the maternal-fetal interface. *J Immunol* **151** : 4562-4573.
- Lindley, I. J. D., Westwick, J. and Kunkel, S. L. (1993). Nomenclature announcement: the chemokines. *Immunol Today* **14** : 24.
- Linnemeyer, P. A. and Pollack, S. B. (1993). Prostaglandin E<sub>2</sub> induced changes in the phenotype, morphology and lytic activity of IL-2 activated natural killer cells. *J Immunol* **150** : 3747-3754.
- Ljunggren, H.-G. and Karre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* **11** : 237-244.
- Loetscher, P., Seitz, M., Clark-Lewis, I., Baggiolini, M. and Moser, B. (1996). Activation of NK cells by CC chemokines - Chemotaxis, Ca<sup>2+</sup> mobilisation and enzyme release. *J Immunol* **156** : 322-327.
- Loke, Y. W. and King, A. (1995a). Uterine mucosal leucocytes. In (Eds) Loke, Y. W. and King, A. *Human Implantation, Cell Biology and Immunology*. Cambridge University Press, Cambridge : 102-129.

- Loke, Y. W. and King, A. (1995b). Human Implantation. Cambridge University Press, Cambridge.
- Loke, Y. W. and King, A. (1996). Immunology of human implantation: an evolutionary perspective. *Hum Reprod* **11** : 283-286.
- Loke, Y. W. and King, A. (1997). Immunology of human placental implantation : clinical implications of our current understanding. *Mol Med Today* **3** : 153-159.
- Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S. and Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci USA* **90** : 11162-11166.
- Ludwig, H. and Spornitz, U. M. (1991). Microarchitecture of the human endometrium by scanning electron microscopy: menstrual desquamation and remodelling. *Ann N Y Acad Sci* **622** : 28-46.
- Lukacs, N. W., Chensue, S. W., Karpus, W. J., Lincoln, P., Keefer, C., Strieter, R. M. and Kunkel, S. L. (1997). CC chemokines differentially alter interleukin-4 production from lymphocytes. *Am J Pathol* **150** : 1861-1868.
- Lumsden, M. A., Brown, A. and Baird, D. T. (1984). Prostaglandin production from homogenates of separated glandular epithelium and stroma from human endometrium. *Prostaglandins* **28** : 485-496.
- Luukkainen, T., Lahteenmaki, P. and Toivonen, J. (1990). Levonorgestrel-releasing intrauterine device. *Ann Med* **22** : 85-90.
- Lydon, J. P., Demayo, F. J., Funk, C. R., Mani, S. K., Hughes, A. R., Montgomery, C. A., Shyamala, O. M., Conneely, O. M. and O'Malley, B. W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev* **9** : 2266-2278.
- Maaskant, R. A., Bogic, L. V., Gilger, S., Kelly, P. A. and Bryant-Greenwood, G. D. (1996). The human prolactin receptor in the fetal membranes, decidua and placenta. *Endocrinology* **81** : 396-405.



- Maathuis, J. B. and Kelly, R. W. (1978). Concentrations of prostaglandin F2 $\alpha$  and E2 in the endometrium throughout the menstrual cycle and after the administration of clomiphene or an oestrogen-progestogen pill and in early pregnancy. *J Endocrinol* **77** : 361-371.
- MacKay, C. R. and Imhof, B. A. (1993). Cell adhesion in the immune system. *Immunol Today* **14** : 99-102
- Maggi, M., Vannelli, G. B., Fantoni, G., Baldie, E., Magini, A., Peri, A., Giannini, S., Gloria, L., Del Carlo, P., Casparis, D. and Tomei, T. (1994). Endothelin in the human uterus during pregnancy. *J Endocrinol* **142** : 385-396.
- Maghazachi, A. A., Al-Aoukaty, A. and Schall, T. J. (1994). C-C Chemokines induce the chemotaxis of NK and IL-2 activated NK cells - role for G proteins. *J Immunol* **153** : 4969-4977.
- Maghazachi, A. A., Al-Aoukaty, A. and Schall, T. J. (1996). CC chemokines induce the generation of killer cells from CD56<sup>+</sup> cells. *Eur J Immunol* **26** : 315-319.
- Maier, J. A. M., Hla, T. and Macaig, T. (1990). Cyclooxygenase is an immediate early gene induced by interleukin-1 in human endothelial cells. *J Biol Chem* **265** : 10805-10808.
- Malter, J. S. (1989). Identification of an AUUUA-specific messenger RNA binding protein. *Science* **246** : 663-666.
- Mangal, R., Weihle, R. D., Poindexter III, A. N., Hilsenrath, R. E. and Weigel, N. L. (1995). Uterine progesterone receptor A and B subunits change in the cycle. *J Soc Gynecol Invest* **2**.
- Mantovani, A., Locati, M., Allavena, P. and Sozzani, S. (1996). The chemokine superfamily : Crosstalk with the IL-1 system. *Immunobiology* **195** : 522-549.
- Marbaix, E., Donnez, J., Courtoy, P. J. and Eeckhout, Y. (1992). Progesterone regulates the activity of interstitial collagenase and related gelatinases A and B in human endometrial explants. *Proc Natl Acad Sci USA* **89** : 11789-11793.
- Marbaix, E., Kokorine, I., Henriët, P., Donnez, J., Courtoy, P. J. and Eeckhout, Y. (1995). The expression of interstitial collagenase in human endometrium is controlled by progesterone and by oestradiol and is related to menstruation.

Markee, J. E. (1940). Menstruation in intraocular endometrial transplants in the rhesus monkey. *Contrib Embryol Carnegie Inst* **177** : 211-308.

Markoff, E., Zeitler, P., Peleg, S. and Handwerger, S. (1983). Characterisation of the synthesis and release of prolactin by an enriched fraction of human decidual cells. *J Clin Endocrinol Metab* **56** : 962-968.

Marsh, M. M., Butt, A. R., Riley, S. C., Rogers, P. A. W., Susil, B., Affandi, B., Findlay, J. K. and Salamonsen, L. A. (1995). Immunolocalisation of endothelin and neutral endopeptidase in the endometrium of users of subdermally implanted levonorgestrel (Norplant). *Hum Reprod* **10** : 2584-2589.

Marsh, M. M., Findlay, J. K. and Salamonsen, L. A. (1996). Endothelin and menstruation. *Hum Reprod* **11** Suppl 2 : 83-89.

Marsh, M. M., Malakooti, N., Taylor, N. H., Findlay, J. K. and Salamonsen, L. A. (1997). Endothelin and neutral endopeptidase in the endometrium of women with menorrhagia. *Hum Reprod* **12** : 2036-2040

Martel, D., Malet, C., Gautray, J. P. and Psychoyos, A. (1981). Surface changes of the luminal uterine epithelium during the human menstrual cycle: a scanning electron microscope study. In (Eds) de Brux, J., Mortel, R. and Guatray, J. P. *The Endometrium: Hormonal Impacts*. Plenum Press, New York.

Martel, D., Monier, M. N., Roche, D. and Psychoyos, A. (1989). Effect of mifepristone (RU486) on concentrations of prostaglandin E<sub>2</sub> binding sites in the rat endometrium. *J Reprod Fertil* **85** : 527-532.

Martel, D., Monier, M. N., Roche, D. and Psychoyos, A. (1991). Hormonal dependence of pinopode formation at the uterine luminal surface. *Hum Reprod* **6** : 597-603.

Marzusch, K., Ruck, P., Geiselhart, A., Handgretinger, R., Dietl, J. A., Kaiserling, E., Horny, H.-P., Vince, G. and Redman, C. W. G. (1993). Distribution of cell adhesion molecules on CD56<sup>++</sup>, CD3<sup>-</sup>, CD16<sup>-</sup> large granular lymphocytes and endothelial cells in first trimester human decidua. *Hum Reprod* **8** : 1203-1208.

- Maslar, I. A. and Riddick, D. H. (1979). Prolactin production by human endometrium during the normal menstrual cycle. *Am J Obstet Gynecol* **14** : 255-261.
- Matrisian, L. M. and Hogan, B. L. M. (1990). Growth factor-regulated proteases and extracellular matrix remodelling during mammalian development. *Curr Top Dev Biol* **24** : 219-259.
- Matsushima, K., Morishita, K., Yoshimura, T., Lavu, S., Kobayashi, Y., Lew, W., Appella, E., Kung, H. F., Leonard, E. J. and Oppenheim, J. J. (1988). Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin-1 and tumour necrosis factor. *J Exp Med* **167** : 1883-1893.
- Matsushima, K. and Oppenheim, J. J. (1989). Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL-1 and TNF. *Cytokine* **1** : 2-13.
- Matsushima, K., Baldwin, E. and Mukaida, N. (1992). Interleukin-8 and MCAF : Novel leukocyte recruitment and activating cytokines. In (Eds) Kishimoto, T. Interleukins : *Molecular Biology and Immunology*. Basel : 236-265.
- McGrath, M. H. (1982). The effect of prostaglandin inhibitors on wound contraction and the myofibroblast. *Plast Reconstr Surg* **69** : 74-83.
- Mertz, P. M., Dewitt, D. L., Stetlerstevenson, W. G. and Wahl, L. M. (1994). Interleukin-10 suppression of monocyte prostaglandin-H synthase-2 - mechanism of inhibition of prostaglandin-dependant matrix metalloproteinase production. *J Biol Chem* **269** : 21322-21329.
- Meyer, M. E., Pornon, A., Ji, J. W. and et al. (1990). Agonistic and antagonistic activities of RU486 on the functions of the human progesterone receptor. *EMBO J* **9** : 3923-3932.
- Middleton, J., Neil, S., Wintle, J., Clark-Lewis, I., Moore, H., Lam, C., Auer, M., Hub, E. and Rot, A. (1997). Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell* **91** : 385-395.
- Ming, W. J., Bersani, L. and Mantovani, A. (1987). Tumour necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. *J Immunol* **138** : 1469-1474.

- Mitchell, M. D. (1992). Biochemistry of the Prostaglandins. *Bailliere's Clin Obstet Gynaecol* **6** : 687-703.
- Mitchell, J. A., Belvisi, M. G., Robbins, R. A., Kwon, O. J., Barnes, P. J. and Vane, J. R. (1994). Induction of cyclooxygenase-2 in human pulmonary epithelial cells. *Br J Pharmacol* **112** : U41
- Morita, I., Schindler, M., Regier, M. K., Otto, J. C., Hori, T., DeWitt, D. L. and Smith, W. L. (1995). Different intracellular locations for prostaglandin endoperoxide synthase-1 and -2. *J Biol Chem* **270** : 10902-10906.
- Mosmann, T. R. and Coffman, R. L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* **7** : 145-173.
- Mosmann, T. R. and Moore, K. W. (1991). The role of IL-10 in crossregulation of TH1 and TH2 responses. *Immunol Today* **12** : A49.
- Mosmann, T. R. and Sad, S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* **17** : 138-146.
- Muccioli, G. and Di Carlo, R. (1994). Modulation of prolactin receptors in the rat hypothalamus in response to changes in serum concentration of endogenous prolactin or to ovine prolactin administration. *Brain Res* **663** : 244-250.
- Mukaida, N., Shiroo, M. and Matsushima, K. (1989). Genomic structure of the human monocyte-derived neutrophil chemotactic factor (MDNCF), interleukin-8. *J Immunol* **143** : 1366-1371.
- Mukaida, N., Hisinuma, A., Zachariae, C. O. C., Oppenheim, J. J. and Matsushima, K. (1990). PMA induces IL-8 mRNA in various cells with kinetics similar to IL-1 / TNF- $\alpha$ . In (Eds) Westwick, J. and et al. *Regulation of IL-8 gene expression and binding of several other members of the intercrine family to receptors of IL-8*. Second International Symposium on Chemotactic Cytokines. Humana Press, Clifton.
- Murphy, L. D., Herzog, C. E., Rudick, J. B., Fojo, A. T. and Bates, S. F. (1990). Use of PCR in the quantitation of *mdr-1* gene expression. *Biochemistry* **29** : 10351-10356.

- Murphy, P. M. (1994). The molecular biology of leukocyte chemoattractant receptors. *Annu Rev Immunol* **12** : 593-633.
- Myatt, L., Bray, M. A., Gordon, D. and Morley, J. (1975). Macrophages on intrauterine devices producing prostaglandin. *Nature* **257** : 227-228.
- Nagase, H. (1991). Matrix Metalloproteinases 1, 2 and 3: substrate specificities and activation mechanisms. In (Eds) Leppert, P. C. and Woessner, J. F. *The extracellular matrix of the uterus, cervix and fetal membranes: synthesis, degradation and hormonal regulation*. Perinatology Press, NY : 28-44.
- Natuzzi, E. S., Ursell, P. C., Harrison, M., Buscher, C. and Riemer, R. K. (1993). Nitric oxide synthase activity in the pregnant uterus decreases at parturition. *Biochem Biophys Res Commun* **194** : 1-8.
- Negus, R. P. M., Stamp, G. W. H., Relf, M. G., Burke, F., Malik, S. T. A., Bernasconi, S., Allavena, P., Sozzani, S., Mantovani, A. and Balkwill, F. R. (1995). The detection and localisation of monocyte chemoattractant protein-1 (MCP-1) in human ovarian cancer. *J Clin Invest* **95** : 2391-2395.
- Neote, K., D, D., Mak, J. K., Horuk, R. and Schall, T. J. (1993). Molecular cloning, functional expression, and signalling characteristics of a C-C chemokine receptor. *Cell* **72** : 415-425.
- Nie, G. Y., Butt, A. R., Salamonsen, L. A. and Findlay, J. K. (1997). Hormonal and non-hormonal agents at implantation as targets for contraception. *Reprod Fertil Dev* **9** : 65-76.
- Niino, H., Otsuka, T., Izuhara, K., Yamaoka, K., Ohshima, K., Tanabe, T., Hara, S., Nemoto, Y., Tanaka, Y., Nakashima, H. and Niho, Y. (1997). Regulation by interleukin-10 and interleukin-4 of cyclooxygenase-2 expression in human neutrophils. *Blood* **89** : 1621-1628.
- Nikas, G., Drakakis, P., Loutradis, D., Mara-Skoufari, C., Koumantakis, E., Michalas, S., Psychoyos, A. (1995). Uterine pinopodes as markers of the 'nidation window' in cycling women receiving exogenous oestradiol and progesterone. *Hum Reprod* **10** : 1208 - 1213.
- Nilsson, C. G., Luukkainen, T., Diaz, J. and et al. (1982). Clinical performance of a new levonorgestrel-releasing intra-uterine device. A randomised comparison with a



Nova T-copper device. *Contraception* **25** : 345-356.

Nilsson, C. G., Lahteenmaki, P. and Luukkainen, T. (1984). Ovarian function in amenorrhoeic and menstruating users of a levonorgestrel-releasing intrauterine device. *Fertil Steril* **41** : 52-55.

Nishino, E., Matsuzaki, N., Masuhiro, K., Kameda, T., Taniguchi, T., Takagi, T., Saji, F. and Tanizawa, O. (1990). Trophoblast derived interleukin-6 (IL-6) regulates human chorionic gonadotropin release through IL-6 receptor on human trophoblasts. *J Clin Endocrinol Metab* **71** : 436-441.

Noyes, R. W., Hertig, A. T. and Rock, J. (1950). Dating the endometrial biopsy. *Fertil Steril* **1** : 3-25.

O'Banion, M. K., Sadowski, H. B., Winn, V. and Young, D. A. (1991). A serum and glucocorticoid regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. *J Biol Chem* **266** : 23267-23267.

O'Banion, M. K., Winn, V. D. and Young, D. A. (1992). cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc Natl Acad Sci USA* **89** : 4888-4892.

O'Reilly, G., Charnock-Jones, D. S., Davenport, A. P., Cameron, I. T. and Smith, S. K. (1992). Presence of messenger ribonucleic acid for endothelin-1, endothelin-2 and endothelin-3 in human endometrium and a change in the ratio of ETA and ETB receptor subtype across the menstrual cycle. *J Clin Endocrinol Metab* **75** : 1545-1549.

Odlind, V. and Fraser, I. S. (1990). Contraception and menstrual bleeding disturbances: a clinical overview. In (Eds) D'Arcangues, C., Fraser, I. S., Newton, J. R. and Odlind, V. *Contraception and mechanisms of endometrial bleeding*. Cambridge University Press, Cambridge : 5-32.

Ohbuchi, H., Nagai, K., Yamaguchi, M., Ikenoue, T., Mori, N., Kitamura, K., Araki, S. and Toshimori, K. (1995). Endothelin-1 and big endothelin-1 in human endometrium during menstruation. *Am J Obstet Gynecol* **173** : 1483-1490.



Oppenheim, J. J., Zachariae, C. O., Mukaida, N. and Matsushima, K. (1991). Properties of the novel proinflammatory supergene 'intercrine' cytokine family. *Annu Rev Immunol* **9** : 617-648.

Ormandy, C. J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N. and Kelly, P. A. (1996). Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev* **11** : 167-178.

Osmers, R. G. W., Bläser, J., Kuhn, W. and Tschesche, H. (1995). Interleukin-8 synthesis and the onset of labor. *Obstet Gynecol* **86** : 223-229.

Pace, D., Morrison, L. and Bulmer, J. N. (1989). Proliferative activity in endometrial stromal granulocytes through the menstrual cycle and early pregnancy. *J Clin Pathol* **42** : 35-39.

Pasmany, L., Mandelboim, O., Valesgomez, M., Davis, D. M., Reyburn, H. T. and Stramihger, J. L. (1996). Protection from natural killer cell-mediated lysis by HLA-G expression on target cells. *Science* **274** : 792-795.

Pekonen, F., Nyman, T., Lahteenmaki, P., Haukkamaa, M. and Rutanen, E.-M. (1992). Intrauterine progestin induces continuous insulin-like growth factor-binding protein-1 production in the human endometrium. *J Clin Endocrinol Metab* **75** : 660-664.

Pellegrini, I., Lebrun, J., Ali, S. and Kelly, P. A. (1992). Expression of prolactin and its receptor in human lymphoid cells. *Mol Endocrinol* **6** : 1023-1031.

Perrot-Applanat, M., Deng, M., Fernandez, H., Lelaidier, C., Meduri, G. and Bouchard, P. (1994). Immunohistochemical localization of estradiol and progesterone receptors in human uterus throughout pregnancy: expression in endometrial blood vessels. *J Clin Endocrinol Metab* **78** : 216-224.

Perrot-Applanat, M., Groyer-Picard, M. T., Garcia, E., Lorenzo, F. and Milgrom, E. (1988). Immunocytochemical demonstration of estrogen and progesterone receptors in muscle cells of uterine arteries in rabbits and humans. *Endocrinology* **123** : 1511-1519.

Perrot-Applanat, M., Gualillo, O., Buteau, H., Edery, M. and Kelly, P. A. (1997). Internalization of prolactin receptor and prolactin in transfected cells does not

involve nuclear translocation. *J Cell Sci* **110** : 1123-1132.

Petterson, K., Grandien, K., Kuiper, G. G. J. M. and Gustafsson, J. A. (1997). Mouse estrogen receptor  $\beta$  forms estrogen response element-binding heterodimers with estrogen receptor  $\alpha$ . *Mol Endocrinol* **11** : 1486-1496.

Philippeaux, M. M. and Piguet, P. F. (1993). Expression of tumour necrosis factor-alpha and its mRNA in the endometrial mucosa during the menstrual cycle. *Am J Pathol* **143** : 480-486.

Philips, J. H., Chang, C., Mattson, J., Gumperz, J. E., Parham, P. and Lanier, L. L. (1996). CD94 and a novel associated protein (94AP) form an NK cell receptor involved in the recognition of HLA-A, HLA-B and HLA-C allotypes. *Immunity* **5** : 163-172.

Pickles, V. R., Hall, W. J., Best, F. A. and Smith, G. N. (1965). Prostaglandins in endometrium and menstrual fluid from normal and dysmenorrhoeic subjects. *J Obstet Gynaecol Br Commonw* **72** : 185.

Pijnenborg, R. (1994). Trophoblast invasion. *Reprod Med Rev* **3** : 53-73.

Polan, M. L., Simon, C., Frances, A., Lee, B. Y. and Prichard, L. E. (1995). Role of embryonic factors in human implantation. *Hum Reprod* **10** Suppl 2 : 22-29.

Poropatich, C., Rojas, M. and Silverberg, S. G. (1987). Polymorphonuclear leukocytes in the endometrium during the normal menstrual cycle. *Int J Gynaecol Pathol* **6** : 230-234.

Posner, B. I., Kelly, P. A. and Friesen, H. G. (1975). Prolactin receptors in rat liver : possible regulation by prolactin. *Science* **188** : 57-59.

Premack, B. A. and Schall, T. J. (1996). Chemokine receptors: gateways to inflammation and infection. *Nat Med* **2** : 1174-1178.

Prigent-Tessier, A., Pageaux, J. F., Fayard, J. M., Lagarde, M., Laugier, C. and Cohen, H. (1996). Prolactin up-regulates prostaglandin E<sub>2</sub> production through increased expression of pancreatic-type phospholipase A<sub>2</sub> (type 1) and prostaglandin G/H synthase 2 in uterine cells. *Mol Cell Endocrinol* **122** : 101-108.

Pritchard Jnr, K. A., O'Banion, M. K., Miano, J. M., Vlasic, N., Bhatia, U. G., Young, D. A. and Stemerman, M. B. (1994). Induction of cyclooxygenase-2 in rat

vascular smooth muscle cells *in vitro* and *in vivo*. *J Biol Chem* **269** : 8504-8509.

Psychoyos, A. (1994). The implantation window: basic and clinical aspects. In (Eds) Mori, T., Aono, T., Tominaga, T. and Hiroi, M. *Perspectives on Assisted Reproduction. Ares-Serono Symposia Series*. Frontiers in Endocrinology, Rome, Italy : 57-63.

Psychoyos, A., Nikas, G. and Gravanis, A. (1995). The role of prostaglandins in blastocyst implantation. *Hum Reprod* **10** Suppl 2 : 30-42.

Raghupathy, R. (1997). Th1-type immunity is incompatible with successful pregnancy. *Immunol Today* **18** : 478-482.

Rajaratnam, K., Sykes, B. D., Kay, C. M., Dewald, B., Geiser, T., Baggiolini, M. and Clark-Lewis, I. (1994). Neutrophil activation by monomeric interleukin-8. *Science* **264** : 90-92.

Rampart, M., Van Damme, J., Zonnekyn, L. and Herman, A. G. (1989). Granulocyte chemotactic protein / interleukin-8 induces plasma leakage and neutrophil accumulation in rabbit skin. *Am J Pathol* **135** : 21-25.

Rankin, J. C., Ledford, B. E., Jansson, H. T. and Baggett, B. (1979). Prostaglandins, indomethacin and the decidual cell reaction in the mouse uterus. *Biol Reprod* **20** : 339-404.

Rao, Ch.V., Chegini, N. and Lei, Z. M. (1989). Immunocytochemical localization of 5- and 12-lipoxygenases and cyclooxygenase in nonpregnant human uteri. In (Eds) Zor, U., Naop, Z. and Danon, A. *New Trends in Lipid Mediators Research*. Karger, Basel : 283-286.

Rees, M. C. P., Parry, D. M., Anderson, A. B. M. and Turnbull, A. C. (1982). Immunohistochemical localisation of cyclooxygenase in the human uterus. *Prostaglandins* **23** : 207-214.

Ristimaki, A., Garfinkel, S., Wessendorf, J., Maciag, T. and Hla, T. (1994). Induction of cyclooxygenase-2 by interleukin-1 $\alpha$ . Evidence for post-transcriptional regulation. *J Biol Chem* **269** : 11769-11775.

Robaye, B., Mosselmans, R., Fiers, W., Dumont, J. E. and Galand, P. (1991). Tumor-necrosis-factor induces apoptosis (programmed cell death) in normal

endothelial cells *in vitro*. *Am J Pathol* **138** : 447-453.

Roberts, D. K., Parmley, T. H., Walker, N. J. and Horbelt, D. V. (1992). Ultrastructure of the microvasculature in the human endometrium throughout the normal menstrual cycle. **166** : 1393-1406.

Rogers, P. A. W., Abberton, K. M. and Susil, B. (1992). Endothelial cell migratory signal produced by human endometrium during the menstrual cycle. *Hum Reprod* **7** : 1061-1066.

Rollins, B. J., Morrison, E. D. and Stiles, C. D. (1988). Cloning and expression of JE, a gene inducible by platelet derived growth factor and whose product has cytokine-like properties. *Proc Natl Acad Sci USA* **85** : 3738-3742.

Rollins, B. J. and Pober, J. S. (1991). Interleukin-4 induces synthesis and secretion of MCP-1/JE by human endothelial cells. *Am J Pathol* **138** : 1315-1319.

Romano, M., Sironi, M., Toniatti, C., Polentarutti, N., Fruscella, P., Ghezzi, P., Faggioni, R., Luini, W., Van Hinsbergh, V., Souani, S., Bussolino, F., Poli, V., Ciliberto, G. and Mantovani, A. (1997). Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity* **6** : 315-325.

Romero, R., Durum, S., Dinarello, C. A., Oyarzun, E., Hobbins, J. C. and Mitchell, M. D. (1989). Interleukin-1 stimulates prostaglandin biosynthesis by human amnion and decidual cells. *Prostaglandins* **37** : 13-22.

Rosselli, M. (1997). Nitric oxide and reproduction. *Mol Hum Reprod* **3** : 639-641.

Roth, S. J., Woldemar Carr, M. and Springer, T. A. (1995). C-C chemokines, but not the C-X-C chemokines interleukin-8 and interferon- $\gamma$  inducible protein-10, stimulate transendothelial chemotaxis of T lymphocytes. *Eur J Immunol* **25** : 3482-3488.

Ryan, I. P., Tseng, J. F., Schriock, E. D., Khorram, O., Landers, D. V. and Taylor, R. N. (1995). Interleukin-8 concentrations are elevated in peritoneal fluid of women with endometriosis. *Fertil Steril* **63** : 929-932.

Sach, M., Bauermeister, K., Burger, J. A., Loetscher, P., Elsner, J., Schollmeyer, P. and Dobos, G. J. (1997). Inverse MCP-1 / IL-8 ratio in effluents of CAPD patients with peritonitis and in isolated cultured human peritoneal macrophages. *Nephrol Dial Transplant* **12** : 315-320.



Saito, S., Morii, T., Enomoto, M., Sakakura, S., Nishikawa, K., Narita, N. and Ichijo, M. (1993a). The effect of interleukin-2 and transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2) on the proliferation and natural killer activity of decidual CD16-CD56<sup>bright</sup> natural killer cells. *Cell Immunol* **152** : 605.

Saito, S., Niskawa, K., Morii, T., Emonoto, M., Narita, N., Motoyoshi, K. and Ichijo, M. (1993b). Cytokine production by CD16-CD56<sup>bright</sup> natural killer cells in the human early pregnancy decidua. *Int Immunol* **5** : 559-563.

Saito, S., Kasahara, T., Sakakura, S., Enomoto, M., Umekage, H., Harada, N., Morii, T., Nishikawa, K., Narita, N. and Ichijo, M. (1994). Interleukin-8 production by CD16-CD56<sup>bright</sup> natural killer cells in the human early pregnancy decidua. *Biochem Biophys Res Commun* **200** : 378-383.

Saito, S., Kasahara, T., Sakakura, S., Umekage, H., Harada, N. and Ichijo, M. (1994). Detection and localization of interleukin-8 mRNA and protein in human placenta and decidual tissues. *J Reprod Immunol* **27** : 161-172.

Salamonsen, L. A. (1996a). Matrix metalloproteinases and their tissue inhibitors in endometrial remodelling and menstruation. *Reprod Med Rev* **5** : 185-203.

Salamonsen, L. A. and Woolley, D. E. (1996b). Matrix metalloproteinases in normal menstruation. *Hum Reprod* **11** Suppl 2 : 124-133.

Salamonsen, L. A., Butt, A. R., Hammind, F. R., Garcia, S. and Zhang, J. (1997). Production of endometrial matrix metalloproteinases, but not their tissue inhibitors, is modulated by progesterone withdrawal in an *in vitro* model for menstruation. *J Clin Endocrinol Metab* **82** : 1409-1415.

Salvemini, D. (1997). Regulation of cyclooxygenase enzymes by nitric oxide. *Cell Mol Life Sci* **53** : 576-582.

Sarafi, M. N., GarciaZepeda, E. A., MacLean, J. A., Charo, I. F. and Luster, A. D. (1997). Murine monocyte chemoattractant protein (MCP)-5: a novel CC chemokine that is a structural and functional homologue of human MCP-1. *J Exp Med* **185** : 99-109.

Sawdy, R., Slater, D., Fisk, N., Edmonds, D. K. and Bennett, P. (1997). Use of a cyclo-oxygenase type-2-selective non-steroidal anti-inflammatory agent to prevent preterm delivery. *Lancet* **350** : 265-266.

- Schall, T. (1997). Fractalkine - a strange attractor in the chemokine landscape. *Immunol Today* **18** : 147.
- Schall, T. and Bacon, K. (1994). Chemokines, Leukocyte Trafficking, and Inflammation. *Curr Opin Immunol* **6** : 865-873.
- Schindler, C. and Darnell Jnr, J. E. (1995). Transcriptional responses to polypeptide ligands: the Jak-Stat pathway. *Annu Rev Biochem* **64** : 621-651.
- Seibert, K. and Masferrer, J. L. (1994). Role of inducible cyclooxygenase (COX-2) in inflammation. *Receptor* **4** : 17-23.
- Seitz, M., Loetscher, P., Dewald, B., Towbin, H., Ceska, M. and Baggiolini, M. (1994). Production of interleukin-1 receptor antagonist, inflammatory chemotactic proteins, and prostaglandin E by rheumatoid and osteoarthritic synoviocytes - regulation by IFN- $\gamma$  and IL-4. *J Immunol* **152** : 2060-2065.
- Sharkey, M. E., Adler, R. R., Nieder, G. L. and Brenner, C. A. (1996). Matrix metalloproteinase expression during mouse peri-implantation development. *Am J Reprod Immunol* **36** : 72-80.
- Shaw, G. and Kamen, R. (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mediates selective mRNA degradation. *Cell* **46** : 659-667.
- Sheppard, B. L. (1987). Endometrial morphological changes in IUD users: a review. *Contraception* **36** : 1-10.
- Shi, W., Mognetti, B., Campana, A. and Bischof, P. (1995). Metalloproteinase secretion by endometrial leukocyte subsets. *Am J Reprod Immunol* **34** : 299-310.
- Shiver, J. W., Su, L. and Henkart, P. A. (1992). Cytotoxicity with target DNA breakdown by rat basophilic leukaemia cells expressing both cytolyisin and granzyme A. *Cell* **71** : 315-322.
- Shrader, W. T. (1993). Insight: subunit functions of the steroid/thyroid receptor family. *Mol Endocrinol* **7** : 1241-1243.
- Shyy, Y. J., Li, Y.-S. and Kolattukudy, P. E. (1990). Structure of human monocyte chemotactic protein gene and its regulation by TPA. *Biochem Biophys Res Commun* **169** : 346-351.



- Shyy, Y.-J., Li, Y.-S., Lin, M.-C. and Chien, S. (1995). Dexamethasone inhibits the shear stress induced MCP-1 gene expression. *FASEB J* **9** : A322.
- Sica, A., Wang, J. M., Colotta, F., Dejana, E., Mantovani, A., Oppenheim, J. J., Larsen, C. G., Zachariae, C. O. C. and Matsushima, K. (1990). Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. *J Immunol* **144** : 3034-3038.
- Silverberg, S. G., Haukkamaa, M., Arko, H., Nilsson, C. G. and Luukkainen, T. (1986). Endometrial morphology during long-term use of levonorgestrel-releasing intrauterine devices. *Int J Gynecol Pathol* **5** : 235-241.
- Simmons, D. L., Xie, W., Chipman, J. G. and Evett, G. E. (1991). Multiple cyclooxygenases: cloning of a mitogen-inducible form. In (Eds) Bailey, J. M. *Prostaglandins, Leukotrienes, Lipoxins and PAF*. Plenum Press, New York.
- Simon, C., Piquette, G. N., Frances, A. and Polan, M. L. (1993). Localisation of interleukin-1 type receptor and interleukin-1 $\beta$  in human endometrium throughout the menstrual cycle. **77** : 549 - 555.
- Simon, C., Frances, A., Piquette, G. N., Eldanasouri, I., Zurawski, G., Dang, W. and Polan, M.L. (1994). Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. *Endocrinology* **134** : 521-528.
- Simon, C., Pellicer, A. and Polan, M. I. (1995a). Interleukin-1 system crosstalk in embryonic implantation. *Hum Reprod* **10** Suppl 1 : 37-48.
- Simon, C., Frances, A., Lee, B. Y., Mercader, A., Huynh, T., Remohi, J., Lake Polan, M. and Pillcer, A. (1995b). Immunohistochemical localisation identification and regulation of the interleukin-1 receptor antagonist in the human endometrium. *Mol Hum Reprod* **1** : 2472-2477.
- Simon, C., Gimeno, M. J., Mercader, A., Frances, A., Velasco, J. G., Remohi, J., Polan, M. L. and Pellicer, A. (1996). Cytokines-adhesion molecules-invasive proteinases. The missing paracrine / autocrine link in embryonic implantation? *Mol Hum Reprod* **2** : 405-424.
- Singh, E. J., Baccarini, I. M. and Zuspan, F. P. (1984). Levels of prostaglandins F<sub>2 $\alpha$</sub>

- and E<sub>2</sub> in human endometrium during the menstrual cycle. *Am J Obstet Gynecol* **121** : 1003-1006.
- Sinosich, M. J., Lee, J., Williams, R. F. and Hodgen, G. D. (1989). RU486 induced suppression of placental neutrophil elastase inhibitor levels. *Placenta* **10** : 569-578.
- Sivin, I., Sanchez, F. and Diaz, S. (1983). Three-year experience with Norplant subdermal conception. *Fertil Steril* **39** : 799-808.
- Sladek, S. M., Regenstein, A. C., Lykins, D. and Roberts, J. M. (1993). Nitric oxide synthase activity in pregnant rabbit uterus decreases on the last day of pregnancy. *Am J Obstet Gynecol* **169** : 1285-1291.
- Slater, D., Berger, L., Moore, G. E. and Bennett, P. R. (1995). Changes in the expression of types 1 and 2 cyclooxygenase in human fetal membranes at term. *Am J Obstet Gynecol* **172** : 77-82.
- Slater, D. M., Dennes, W. and Jones, G. D. (1997). Expression of COX-1 and COX-2 in human myometrium: changes in relation to gestational age and labour onset. *J Soc Gynecol Invest* **4** Suppl : 115A.
- Smith, S. K., Abel, M. H., Kelly, R. W. and Baird, D. T. (1981). Prostaglandin synthesis in the endometrium of women with ovular dysfunctional bleeding. *Br J Obstet Gynecol* **88** : 434-442.
- Smith, S. K. and Kelly, R. W. (1987). The effect of antiprogestins RU 486 and ZK 98,734 on the synthesis, and metabolism of PGF<sub>2α</sub> and PGE<sub>2</sub> in separated cells from early human decidua. *J Clin Endocrinol Metab* **63** : 527-537.
- Smith, S. K. and Kelly, R. W. (1988). The release of PGF<sub>2α</sub> and PGE<sub>2</sub> from separated cells of human endometrium and decidua. *Prostaglandins Leukot Essent Fatty Acids* **33** : 91-96.
- Smith, S. K. (1996). Vascular endothelial growth factor and the endometrium. *Hum Reprod* **11** Suppl 2 : 56-61.
- Snijders, M. P., de Goeij, A. F., Debets-Te Baerts, M. J. C., Rousch, M. J., Koudstaal, J. and Bosman, F. T. (1992). Immunocytochemical analysis of oestrogen receptors and progesterone receptors in the human uterus throughout the menstrual

cycle and after the menopause. *J Reprod Fertil* **94** : 363 - 371.

Snijdwint, F. G. M., Kalinski, P., Wierenga, E. A., Bos, J. D. and Kapsenberg, M. L. (1993). Prostaglandin E<sub>2</sub> differentially modulates cytokine secretion profiles of human T helper lymphocytes. *J Immunol* **150** : 5321-5329.

Somkuti, S. G., Sun, J., Yowell, C. W., Fritz, M. A. and Lessey, B. A. (1996). The effect of oral contraceptive pills on markers of endometrial receptivity. *Fertil Steril* **65** : 484-488.

Springer, T.A. (1990). Adhesion receptors of the immune system. *Nature* **346** : 425-434.

Standiford, T. J., Strieter, R. M., Chensue, S. W., Westwick, J., Kasahara, K. and Kunkel, S. L. (1990). Interleukin-4 inhibits the expression of interleukin-8 from stimulated human monocytes. *J Immunol* **145** : 1435-1439.

Staples, L. D., Heap, R. B., Wooding, F. B. P. and King, G. J. (1983). Migration of leukocytes into the uterus after acute removal of ovarian progesterone during early pregnancy in the sheep. *Placenta* **4** : 339-350.

Starkey, P., Clover, L. and Rees, M. (1991). Variation during the menstrual cycle of immune cell populations in human endometrium. *Eur J Obstet Gynaecol Reprod Biol* **39** : 203-207.

Stewart, C. L., Karpur, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F. and Abbondanzo, S. J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359** : 76-79.

Stewart, C. L. (1994). The role of leukaemia inhibitory factor (LIF) and other cytokines in regulating implantation in mammals. *Ann N Y Acad Sci* **734** : 157-165.

Stoeckle, M. Y. (1991). Posttranscriptional regulation GRO-alpha, GRO-beta, GRO-gamma and IL-8 messenger RNA by IL-1-beta. *Nucl Acids Res* **19** : 917-920.

Strahle, U., Boshart, M., Klock, G., Stewart, F. and Schütz, G. (1989). Glucocorticoid and progesterone specific effects are determined by differential expression of the respective hormone receptors. *Nature* **339** : 629-632.

Strassmann, G., Patil Koota, V., Finkelman, F., Fong, M. and Kambayashi, T. (1994). Evidence for the involvement of interleukin 10 in the differential

- deactivation of murine peritoneal macrophages by prostaglandin E<sub>2</sub>. *J Exp Med* **180** : 2365-2370.
- Strauss III, J. F. and Gurside, E. (1991). The Endometrium : Regulation and Dysfunction. In (Eds) Yen, S. S. C. and Jaffe, R. B. *Reproductive Endocrinology - Physiology, Pathophysiology and Clinical Management*. W B Saunders Co. : 309-338.
- Stray-Pederson, B. and Stray-Pederson, S. (1984). Etiological factors and subsequent reproductive performance in 195 couples with a prior history of habitual abortion. *Am J Obstet Gynecol* **148** : 140-146.
- Strieter, R. M., Kunkel, S. L., Showell, H. J., Remick, D. G., Phan, S. H., Ward, P. A. and Marks, R. M. (1989). Endothelial cell gene expression of a neutrophil chemotactic factor by TNF- $\alpha$ , LPS, and IL-1 $\beta$ . *Science* **243** : 1467-1469.
- Strieter, R. M., Kunkel, S. L., Elner, V. M., Martonyi, C. L., Koch, A. E., Polverini, P. J. and Elner, S. G. (1992). IL-8: a corneal factor that induces neovascularisation. *Am J Pathol* **141** : 1279-1284.
- Strieter, R. M., Polverini, P. J., Kunkel, S. L., Arenberg, D. A., Burdick, M. D., Kasper, J., Dzuiba, J., Van Damme, J., Walz, A., Marriott, D., Chan, S. Y., Rocznia, S. and Shanafelt, A. B. (1995a). The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J Biol Chem* **270** : 27348-27357.
- Strieter, R. M., Polverini, P. J., Arenberg, D. A., Walz, A., Opdenakker, G., Van Damme, J. and Kunkel, S. L. (1995b). Role of C-X-C chemokines as regulators of angiogenesis in lung cancer. *J Leuk Biol* **57** : 752-762.
- Strieter, R. M., Standiford, T. J., Huffnagle, G. B., Colletti, L. M., Lukacs, N. W. and Kunkel, S. L. (1996). "The good, the bad, and the ugly" - the role for chemokines in models of human disease. *J Immunol* **156** : 3583-3586.
- Sueoka, K., Shiokawa, S., Miyazaki, T., Kuji, N., Tanaka, M. and Yoshimura, Y. (1997). Integrins and reproductive physiology: expression and modulation in fertilization, embryogenesis and implantation. *Fertil Steril* **67** : 799-811.
- Sutherland, A. E., Calarco, P. G. and Damsky, C. H. (1993). Developmental

- regulation of integrin expression at the time of implantation in the mouse embryo. *Development* **119** : 1175-1186.
- Swahn, M. L. and Bygdeman, M. (1987). Interruption of early gestation with prostaglandin and antiprogestin. In (Eds) Diczfalusy, E. and Bygdeman, M. *Fertility Regulation Today and Tomorrow*. Raven, NY : 109-118.
- Swahn, M. L., Bygdeman, M., Cekan, S., Xing, S., Masironi and Johannisson, E. (1990). The effects of RU486 administered during the early luteal phase on bleeding pattern, hormonal parameters and endometrium. *Hum Reprod* **5** : 402 - 408.
- Swahn, M. L., Johannisson, E., Daniore, V., de la Torres, B. and Bygdeman, M. (1988). The effect of RU486 administered during the proliferative and secretory phases of the cycle on the bleeding pattern, hormonal parameters and the endometrium. *Hum Reprod* **3** : 915-921.
- Swierkosz, T. A., Mitchell, J. A., Warner, T. D., Botting, R. M. and Vane, J. R. (1995). Coinduction of nitric oxide synthase and cyclooxygenase - interactions between nitric oxide and prostanoids. *Br J Pharmacol* **114** : 1335-1342.
- Tabibzadeh, S. S., Santhanam, U., Sehgal, P. B. and May, L. T. (1989a). Cytokine induced production of IFN- $\beta$ 2 by freshly explanted human endometrial stromal cells. Modulation by estradiol-17 $\beta$ . *J Immunol* **142** : 3134-3139.
- Tabibzadeh, S. S., Santhanam, U., May, L. and Sehgal, P. (1989b). Cytokine induced production of IL-6 by freshly explanted human endometrial stromal cells - modulation by estradiol-17-beta. *Ann N Y Acad Sci* **557** : 543-544.
- Tabibzadeh, S. S. (1990). Proliferative activity of lymphoid cells in human endometrium throughout the menstrual cycle. *J Clin Endocrinol Metab* **70** : 437-443.
- Tabibzadeh, S. S. (1991). Human endometrium: an active site of cytokine production and action. *Endocr Rev* **12** : 272 - 290.
- Tabibzadeh, S. S. and Sun, X. Z. (1992). Cytokine expression in human endometrium throughout the menstrual cycle. *Hum Reprod* **7** : 1214-1221.
- Tabibzadeh, S. S. (1994a). Role of cytokines in endometrium and at the fetomaternal interface. *Reprod Med* **3** : 11-28.



Tabibzadeh, S. S. (1994b). Cytokines and the hypothalamic-pituitary-ovarian-endometrial axis. *Hum Reprod* **9** : 947-967.

Tabibzadeh, S. S., Kong, Q. F., Satyaswaroop, P. G., Zupi, E., Marconi, D., Romanini, C. and Kapur, S. (1994c). Distinct regional and menstrual cycle dependent distribution of apoptosis in human endometrium. Potential regulatory role of T cells and TNF $\alpha$ . *Endocr J* **2** : 87-95.

Tabibzadeh, S. and Babaknia, A. (1995a). The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion. *Mol Hum Reprod* **1** : 1579-1602.

Tabibzadeh, S. S., Kong, O. F., Babaknia, A. and May, L. T. (1995b). Progressive rise in the expression of interleukin-6 in human endometrium during the menstrual cycle is initiated during the implantation window. *Hum Reprod* **10** : 2793-2799.

Tadokoro, N., Koibuchi, N., Ohtake, H., Kawatsu, T., Tanaka, S., Ohkawa, H., Kato, Y., Yamaoka, S. and Kumasaka, T. (1995). Localisation of prolactin and its receptor messenger RNA in the human decidua. *Experientia* **51** : 1216-1219.

Tanaka, Y., Adams, D. and Shaw, S. (1993). Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol Today* **14** : 111-115.

Tarvares, I. A., Bishai, P. and Bennett, A. (1995). Activity of nimesulide on constitutive and inducible cyclo-oxygenases. *Arzneim Forsch Drug Res* **45** : 1093-1095.

Taub, D. D., Lloyd, A. R., Conlon, K., Wang, J. M., Ortaldo, J. R., Harada, A., Matsushima, K., Kelvin, D. J. and Oppenheim, J. J. (1993). Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J Exp Med* **177**

Telfer, J. F., Lyall, F., Norman, J. E. and Cameron, I. T. (1995). Identification of nitric oxide synthase in human uterus. *Hum Reprod* **10** : 19-23.

Telfer, J. F., Irvine, G. A., Kohnen, G., Campbell, S. and Cameron, I. T. (1997).



- Expression of endothelial and inducible nitric oxide synthase in non-pregnant and decidualised human endometrium. *Mol Hum Reprod* **3** : 69-75.
- Tetsuka, T., Daphnaiken, I. D., Srivastava, S. K., Baier, L. D., Du Maine, J. and Morrison, A. R. (1994). Cross talk between cyclooxygenase and nitric oxide pathways - prostaglandin E<sub>2</sub> negatively modulates induction of nitric oxide synthase by interleukin-1. *Proc Natl Acad Sci USA* **91** : 12168-12172.
- Townson, D. H., Remick, D. G., Warren, J. S. and Keyes, P. L. (1996). The effect of dexamethasone (DEX) on prolactin induced monocyte chemoattractant protein-1 (MCP-1) expression and macrophage infiltration in regressing corpora lutea of the rat. *Biol Reprod* **54** Suppl 1: 354.
- Tsao, P. S., Wang, B. Y., Buitrago, R., Shyy, J. Y. J. and Cooke, J. P. (1997). Nitric oxide regulates monocyte chemotactic protein-1. *Circulation* **96** : 934-940.
- Tung, L., Mohamed, M. K., Hoeffler, J. P., Takimoto, G. S., Horwitz, K. B. (1993). Antagonist-occupied human progesterone B-receptors actuate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors. *Mol Endocrinol* **7** : 1256-1265.
- Uguccioni, M., D'Apuzzo, M., Loetscher, M., Dewald, B. and Bagglioni, M. (1995). Actions of the chemotactic cytokines MCP-1, MCP-2, MCP-3, RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  on human monocytes. *Eur J Immunol* **25** : 64-68.
- Uguccioni, M., Loetscher, P., Forssmann, U., Dewald, B., Li, H. D., Lima, S. H., Li, Y. L., Kreider, B., Garotta, G., Thelen, M. and Bagglioni, M. (1996). Monocyte chemotactic protein-4 (MCP-4), a novel structural and functional analogue of MCP-3 and eotaxin. *J Exp Med* **183** : 2379-2384.
- Valentich, J. D., Saada, J. I. and Powell, D. W. (1994). Interleukin-1 $\alpha$  (IL-1) stimulates prostaglandin H synthase (PGHS) gene expression in a human colonic myofibroblast-like cell line (18Co). *Gastroenterology* **106** SS : A787.
- van der Burg, B., Liden, J., Okret, S., Delaunay, F., Wissink, S., van der Saag, P. T. and Gustafsson, J.-A. (1997). Nuclear factor- $\kappa$  B repression in anti-inflammatory

and immunosuppression by glucocorticoids. *Trends Endocrinol Metab* **8** : 152-157.

van der Weiden, R. M. F., Helmerhorst, F. M. and Keirse, M. J. N. C. (1991). Influence of prostaglandins and platelet activating factor on implantation. *Hum Reprod* **6** : 436-442.

van Look, P. F. A. and Bygdeman, M. (1989). Antiprogestational steroids: a new dimension in human fertility regulation. In (Eds) Milligan, S. R. *Oxford Reviews of Reproductive Biology*. Oxford University Press, Oxford : 1-60.

Vane, J. R., Mitchell, J. A., Appleton, I., Tomlinson, A., Bishopbailey, D., Croxtall, J. and Willoughby, D. A. (1994). Inducible isoforms of cyclooxygenase and nitric oxide synthase in inflammation. *Proc Natl Acad Sci USA* **91** : 2046-2050.

Vane, J. R. and Botting, R. M. (1995). New insights into the action of anti-inflammatory drugs. *Inflamm Res* **44** : 1-10.

Vassiliadou, N. and Bulmer, J. N. (1996). Immunohistochemical evidence for increased numbers of 'classic' CD57<sup>+</sup> natural killer cells in the endometrium of women suffering spontaneous early pregnancy loss. *Hum Reprod* **11** : 1569-1574.

Vince, G. S. and Johnson, P. M. (1996). Is there a Th2 bias in human pregnancy? *J Reprod Immunol* **32** : 101-104.

Vincent, A. J., Malakooli, N., Affandi, B., Rogers, P. A. W. and Salamonsen, L. A. (1997). Matrix metalloproteinase-9 and migratory cells are associated with abnormal uterine bleeding in women using Norplant. *Proc Aust Soc Reprod Biol* **27** : 60.

Wang, H., Critchley, H. O. D., Kelly, R. W., Davie, J., Mayo, A., Baird, D. T. and Glassier, A. F. (1997a). Does the once a month administration of an anti-progestogen improve bleeding patterns among women using Norplant? *Hum Reprod* **12** Abstract Book 1 : 179-180.

Wang, H., Critchley, H. O. D., Kelly, R. W., Shen, D. Y. and Baird, D. T. (1997b). Progesterone receptor subtype B is differentially regulated in human endometrial stroma. *Mol Human Reprod* **In Press**.

Wang, J. M., Sica, A., Peri, G., Walter, S., Padura, I. M., Libby, P., Ceska, M., Lindley, I., Colotta, F. and Mantovani, A. (1991). Expression of monocyte

- chemotactic protein-1 and interleukin-8 by cytokine-activated human vascular smooth muscle cells. *Arterioscler Thromb* **11** : 1166.
- Wang, J. D., Fu, Y., Shi, W. L., Zhu, P., Cheng, J., Qiao, G., Wang, Y. and Greene, G. L. (1992). Immunohistochemical localisation of progesterone receptor in human decidua of early pregnancy. *Hum Reprod* **7** : 123-127.
- Warmington, K. S., Ruth, J. H., Sanghi, P. S., Kunkel, S. L. and Chensue, S. W. (1996). Differential expression of monocyte chemoattractant protein-1 (MCP-1) in TH1 and TH2 cytokine-mediated granuloma formation. *FASEB J* **10** : 42.
- Watson, C. J. and Burdon, T. G. (1996). Prolactin signal transduction mechanisms in the mammary gland: the role of the Jak/Stat pathway. *Rev Reprod* **1** : 1-5.
- Wegmann, T. G., Lin, H., Guilbert, L. and Mosmann, T. R. (1993). Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today* **14** : 353-356.
- Wells, T. N. C., Power, C. A., Lusti-Narasimhan, M., Hoogewerf, A. J., Cooke, R. M., Chung, C.-W., Peitsch, M. C. and Proudfoot, A. E. I. (1996a). Selectivity and antagonism of chemokine receptors. *J Leuk Biol* **59** : 53-60.
- Wells, T. N. C., Proudfoot, A. E. I., Power, C. A. and Marsh, M. (1996b). Chemokine receptors - the new frontier for AIDS research. *Chem Biol* **3** : 603-609.
- Williams, T. J. and Morley, J. (1973). Prostaglandins as potentiators of increased vascular permeability in inflammation. *Nature* **246** : 215-217.
- Willis, A. L. and Smith, D. L. (1994). Metabolism of arachidonic acid: an overview. In (Eds) Cunningham, F. M. *Lipid mediators*. Academic Press, London : 2-32.
- Witt, D. P. and Lander, A. D. (1994). Differential binding of chemokines to glycosaminoglycan subpopulations. *Curr Biol* **4** : 394-400.
- Wood, G. W., Hausman, E. and Choudhuri, R. (1997). Relative role of CSF-1, MCP-1/JE and RANTES in macrophage recruitment during successful pregnancy. *Mol Reprod Dev* **46** : 62-70.
- Wu, W. X., Glasier, A., Norman, J., Kelly, R. W., Baird, D. T. and McNeilly, A. S. (1990). The effects of the antiprogesterin mifepristone, *in vivo*, and progesterone *in vitro* on prolactin production by the human decidua in early pregnancy. *Hum*

Wu, W.-X., Brooks, J., Millar, M. R., Ledger, W. L., Saunders, P. T. K., Glasier, A. F. and McNeilly, A. S. (1991). Localisation of the sites of synthesis and action of prolactin by immunocytochemistry and *in situ* hybridisation within the human utero-placental unit. *J Mol Endocrinol* **7** : 241-247.

Wu, W.-X., Brooks, J., Glasier, A. F. and McNeilly, A. S. (1995). The relationship between decidualisation and prolactin mRNA and production at different stages of human pregnancy. *J Mol Endocrinol* **14** : 255-261.

Wu, H., Devi, R. and Malarkey, W. B. (1996). Expression and localization of prolactin messenger ribonucleic acid in the human immune system. *Endocrinology* **137** : 349-353.

Xie, W., Chipman, J. G., Robertson, D. L., Erikson, R. L. and Simmons, D. L. (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci USA* **88** : 2692-2696.

Yen, S. S. C. (1991). The human menstrual cycle. In (Eds) Yen, S. S. C. and Jaffe, R. B. *Reproductive Endocrinology, Physiology, Pathophysiology and Clinical Management*. WB Saunders Company, Harcourt Brace Jovanovich Inc., London.

Yong, E. L., Glasier, A. F., Hillier, H., Ledger, W., Caord, L., Beattie, G., Sweeting, V., Thong, J. and Baird, D. T. (1992). Effect of cyclofenil on hormonal dynamics, follicular development and cervical mucus in normal and oligomenorrhoeic women. *Hum Reprod* **7** : 39-43.

Yoshic, O., Imai, T. and Nomiyama, H. (1997). Novel lymphocyte-specific CC chemokines and their receptors. *J Leuk Biol* **62** : 634-644.

Yoshimura, T. and Leonard, E. J. (1990). Secretion by human fibroblasts of monocyte chemoattractant protein-1, the product of the gene JE. *J Immunol* **144** : 2377-2383.

Yoshimura, T., Yuhki, N., Moore, S. K., Apella, E., Lerman, M. I. and Leonard, E. J. (1989). Human monocyte chemoattractant protein-1 (MCP-1). Full length cDNA

cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to a mouse competence gene JE. *FEBS Lett* **244** : 487.

Yu-Lee, L.-Y. (1997). Molecular actions of prolactin in the immune system. *Proc Soc Exp Biol Med* **215** : 35-48.

Yue, T. L., Wang, X., Sung, C. P., Olson, B., McKenna, P. J., Gu, J. L. and Feuerstein, G. Z. (1994). Interleukin-8, a mitogen and chemoattractant for vascular smooth muscle cells. *Circulation Res* **75** : 1-7.

Zarmakoupis, P. N., Rier, S. E., Maroulis, G. B. and Becker, J. L. (1995). Inhibition of human endometrial stromal cell proliferation by interleukin 6. *Hum Reprod* **10** : 2395-2399.

Zeihner, A., Fisslthaler, B., Schray-Utz, B. and Busse, R. (1995). Nitric oxide modulates the expression of monocyte chemoattractant protein-1 in cultured human endothelial cells. *Circulation Res* **76** : 980-986.

Zullo, J. N., Cochran, B. H., Huang, A. S. and Stiles, C. D. (1985). Platelet derived growth factor and double stranded ribonucleic acids stimulate the expression of the same genes in 3T3 cells. *Cell* **43**

Zuo, J., Lei, Z. M., Rao, Ch. V., Pietrantoni, M. and Cook, V. D. (1994). Differential cyclooxygenase-1 and -2 gene expression in human myometria from preterm and term deliveries. *J Clin Endocrinol Metab* **79** : 894-899.

## APPENDIX I

### Recipes for Solutions

All chemicals were purchased from Merck Ltd. (BDH), Lutterworth, UK unless stated otherwise:

#### **10% Neutral Buffered Formalin (NBF)**

6.5g	$\text{Na}_2\text{HPO}_4$
4.52g	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$
100mls	Formaldehyde
900mls	distilled $\text{H}_2\text{O}$

#### **0.01M Sodium Citrate Buffer pH 6.0**

2.94g	tri-sodium citrate
1 Litre	distilled $\text{H}_2\text{O}$

#### **0.1M Phosphate Buffered Saline (PBS) pH 7.4 - 7.6**

1.26g	$\text{Na}_2\text{HPO}_4$
0.26g	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$
7.6g	$\text{NaCl}$
1 litre	distilled $\text{H}_2\text{O}$

#### **PBS-Tween (PBS-T) pH 7.4 - 7.6**

1 litre of PBS supplemented with 100 $\mu\text{l}$  Tween-20 and 8g  $\text{NaCl}$

#### **Tris Buffered Saline (TBS) pH 7.4 - 7.6**

0.9g	Trisma Base
6.85g	Tris-HCl
8.78g	$\text{NaCl}$
1 litre	distilled $\text{H}_2\text{O}$

#### **TBS-Tween (TBS-T) pH 7.4 - 7.6**

1 litre of TBS supplemented with 100 $\mu\text{l}$  Tween-20 and 8g  $\text{NaCl}$



**ELISA Buffer**                      **pH 7.2**

100mM	Tris	
0.125g	2-methylisotiazolone	(Boehringer Mannheim, Sussex, UK)
0.125g	Bromonitrodioxane	(Boehringer Mannheim, Sussex, UK)
2mg	Bovine Serum Albumin (fatty acid free)	(Sigma, Poole, UK)
300µl	NaCl	(Sigma, Poole, UK)
2mM	EDTA	(Sigma, Poole, UK)
1.5mls	20% Tween-20	
1 litre	distilled H <sub>2</sub> O	

**ELISA Wash Buffer**                      **pH 7.0 - 7.5**

250µl	Tween-20
4.5g	NaCl
1 litre 10mM	Tris

**ELISA Peroxidase Substrate**

10mls	100mM Sodium Acetate (pH 6.0)
1ml	Tetramethylbenzidine in DMF (3mg/ml)
1ml	0.5% Urea hydrogen peroxide in 50mM Acetate pH 6.0

**PBS-BSA**

100mls	Sterile PBS	(Gibco)
0.1g	BSA	(Sigma, Poole, UK)
0.1ml	Preservative	(Boehringer Mannheim, Sussex, UK)

**ELISA Denaturing Solution**

0.5mls	10N NaOH	(100mM)
0.2mls	0.5M EDTA	(2mM)
49.3mls	distilled H <sub>2</sub> O	

**10 x TBE Gel Running Buffer**

108g	Trisma Base
55g	Boric Acid
5.8g	EDTA
1 litre	distilled H <sub>2</sub> O

Diluted 1 in 20 for working dilution of 0.5 X TBE

**Gel Loading Sample Buffer**

3mls	10 X TBE buffer
3.2mls	Glycerol
0.3mls	Bromophenol blue
0.3mls	1% Xylene Cyanol
3.2mls	distilled H <sub>2</sub> O

## APPENDIX II

### Detailed protocols for RT-PCRs

#### Reverse Transcriptase reaction mix for GAP-DH:

11.5µl	DEPC H <sub>2</sub> O
4µl	5 x reverse transcriptase buffer
1µl	dNTPs (premixed, 10mM each dNTP)
1µl	Biotinylated GAP-DH antisense oligonucleotide primer (10µM)
0.25µl	RNAsin (RNase inhibitor)
0.25µl	AMV Reverse transcriptase

#### PCR reaction mix for GAP-DH:

20.5µl	DEPC H <sub>2</sub> O
5µl	10 x PCR buffer
3µl	MgCl <sub>2</sub>
1µl	GAP-DH sense oligonucleotide primer (10µM)
0.5µl	AmpliTaq Polymerase (5U/µl)

Differences in reaction mixes for IL-8, MCP-1, COX-2 and PRL-R:

#### Reverse Transcriptase reaction mix for IL-8:

0.8µl	Biotinylated IL-8 antisense oligonucleotide primer
-------	--

#### PCR reaction mix for IL-8:

0.8µl	IL-8 sense oligonucleotide primer
-------	-----------------------------------

### MCP-1

#### Reverse Transcriptase reaction mix for MCP-1:

0.3µl	Biotinylated MCP-1 antisense oligonucleotide primer
-------	---

#### PCR reaction mix for MCP-1:

0.8µl	MCP-1 sense oligonucleotide primer
-------	------------------------------------

## **COX-2**

### **Reverse Transcriptase reaction mix for COX-2:**

0.8μl            Biotinylated COX-2 antisense oligonucleotide primer

### **PCR reaction mix for COX-2:**

0.8μl            COX-2 Sense oligonucleotide primer

2μl            MgCl<sub>2</sub>

## **PRL-R**

### **Reverse Transcriptase reaction mix for PRL-R:**

1μl            PRL-R antisense oligonucleotide primer

### **PCR reaction mix for PRL-R:**

1μl            PRL-R sense oligonucleotide primer

## CONFERENCE PRESENTATIONS

**Jones, R.L.**, Critchley, H.O.D., Kelly, R.W. & Baird, D.T. (1997) Downregulation of IL-8 and COX-2 by progesterone in human endometrium. *Journal of Reproduction and Fertility*, Abstract series 20, p14.

**Jones, R.L.** (1997) Immunomodulatory agent in peri-implantation human endometrium. Blair Bell Jubilee Competition, Royal College of Obstetricians and Gynaecologists, London, December 1997.

(Theodore Ogg Prize winner, chosen to represent Edinburgh University at the Blair Bell Jubilee Competition).

Critchley, H.O.D., **Jones, R.L.**, Wang, H., Kelly, R.W., McNeilly, A.S., Gebbie, A.E. & Glasier, A.F. (1997) Endometrial sex steroid and prolactin-receptor localisation following intra-uterine levonorgestrel delivery. *Human Reproduction*, 12, Abstract Book 1, P-228

**Jones, R.L.**, Critchley, H.O.D. & Kelly, R.W. (1996) Chemokine Expression in Human Endometrium. *Journal of British Fertility Society 2: (1) Human Reproduction*, Natl. Suppl. 6A (S14)

Critchley, H.O.D., Lea, R.G., Kelly, R.W., **Jones, R.L.**, Drudy, T.A. & Baird, D.T. (1996) Antigestogen administration increases macrophage influx into first trimester human decidua. *Journal of Endocrinology*, 148 (Suppl). Abstract No. P22

Critchley, H.O.D., Lea, R.G., **Jones, R.L.**, Cameron, S.T., Gebbie, A.E. & Glasier, A.F. (1995) The effect of continuous intra-uterine levonorgestrel upon endometrial sex steroid receptor distribution, interleukin-8 expression and leukocyte populations. *Journal of Endocrinology*, 144 (Suppl) P136

**Jones, R.L.**, Critchley, H.O.D., Jabbour, H.N., Brooks, J. & McNeilly, A.S. (1996) Prolactin receptor expression in human endometrium. 8th Simpson Symposium, Edinburgh, Scotland, August 1996  
(Poster Presentation - won first prize for best poster).

**Jones, R.L.**, Kelly, R.W. & Critchley, H.O.D. (1996) Immunolocalisation of monocyte chemoattractant protein-1 (MCP-1) and Cyclooxygenase-2 (COX-2) in human endometrium and decidua. 4th European Congress on Prostaglandins and

other Locally Active Factors in Reproduction. Stockholm. Sweden. Free Communication F1.

Critchley, H.O.D., Glasier, A.F., Gebbie, A. E., **Jones, R.L.**, Cameron, S.T., Carr, G.C. & Kelly, R.W. (1996) Anomalous endometrial prostaglandin (PG) metabolism with local intrauterine levonorgestrel delivery. 4th European Congress on Prostaglandins and other Locally Active Factors in Reproduction. Stockholm. Sweden. Free Communication P5.



## PUBLICATIONS

**Jones, R.L.**, Critchley, H.O.D., Brooks, J., Jabbour, H. N. & McNeilly, A.S. (1998) Localisation and Temporal Expression of Prolactin Receptor in Human Endometrium. *Journal of Clinical Endocrinology and Metabolism*. In Press.

**Jones, R.L.**, Kelly, R.W. & Critchley, H.O.D. (1997) Chemokine and Cyclooxygenase-2 Expression in Human Endometrium Coincides with Leukocyte Accumulation. *Human Reproduction*, 12 (6) 1300 - 1306.

Critchley, H.O.D., **Jones, R.L.**, Lea, R.G., Drudy, T.A., Kelly, R.W., Williams, A.R. & Baird, D.T. (1997) The role of progesterone in expression of inflammatory mediators in human endometrium. *Journal of Clinical Endocrinology and Metabolism*. Submitted.

Critchley, H.O.D., Wang, H, **Jones, R.L.**, Kelly, R.W., Drudy, T.A., Gebbie, A.E., Buckley, C.H., McNeilly, A.S. & Glasier, A.F. (1997) Morphological and functional features of endometrial decidualisation following long term intra uterine levonorgestrel delivery. *Human Reproduction*. Submitted.

Critchley, H.O.D., Kelly, R.W., Lea, R.G., Drudy, T.A., **Jones, R.L.** & Baird, D.T. (1996) Sex steroid regulation of leukocyte traffic in human decidua. *Human Reproduction*. 11, 2257-2262.

Baird, D.T., Cameron, S.T., Critchley, H.O.D., Drudy, T.A., Howe, A., **Jones, R.L.**, Lea, R.G. & Kelly, R.W. (1996) Prostaglandins and menstruation. *European Journal of Obstetrics and Gynaecology and Reproductive Biology*. 70, 15-17

## Sex steroid regulation of leukocyte traffic in human decidua

H.O.D.Critchley<sup>1,3</sup>, R.W.Kelly<sup>2</sup>, R.G.Lea<sup>1</sup>,  
T.A.Drudy<sup>1</sup>, R.L.Jones<sup>1</sup> and D.T.Baird<sup>1</sup>

<sup>1</sup>Department of Obstetrics and Gynaecology, University of Edinburgh and <sup>2</sup>Medical Research Council, Reproductive Biology Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9EW, UK

<sup>3</sup>To whom correspondence should be addressed

Endometrial leukocyte subpopulations vary over the reproductive cycle, but no data exist on the mechanism regulating their recruitment into uterine tissue. This study has evaluated the role of progesterone in the recruitment of selected leukocyte populations in early pregnancy decidua. Decidua was collected from women in early pregnancy at the time of vacuum aspiration of the uterus 6, 12, 24 and 36 h after taking 200 mg mifepristone (RU486). Standard immunohistochemical techniques were employed to demonstrate the selected leukocyte populations in decidual tissue and these were analysed using image analysis. Fresh decidua was incubated in medium for 24 h and supernatants assayed for interleukin (IL)-8 (neutrophil chemotactic factor) and MCP-1 (monocyte chemoattractant protein-1) content. Analysis of variance demonstrated a significant increase in tissue monocyte number in decidua 12–36 h after mifepristone administration. No significant changes in other leukocyte subpopulations were observed. Decidual IL-8 concentrations were significantly increased ( $P = 0.019$ ) 6 h after mifepristone and decidual MCP-1 concentration rose (non-significant) and fell significantly ( $P = 0.029$ ) between 6 and 12 h after mifepristone. Progesterone withdrawal may initiate a local cascade of events involving inflammatory mediators which in turn are responsible for the influx of monocytes. This influx may be essential in the process of shedding of endometrium or decidua since monocytes and neutrophils are important sources of proteases and collagenases. Furthermore, these cells are potential local sources of immunomodulatory cytokines.

**Key words:** antigestogens/decidua/leukocytes/progesterone

### Introduction

Although the precise local mechanisms controlling implantation, decidualization and menstruation are unknown, there are several lines of evidence to suggest that interaction between steroid hormones, leukocytes and locally produced cytokines and growth factors are involved. The local tissue response to withdrawal of progesterone resulting in menstruation shows many features characteristic of an inflammatory response, that

is release of prostaglandins, increased permeability of blood vessel walls and an influx of leukocytes in the endometrium (Kelly, 1994). The number and type of leukocytes in human endometrium and decidua varies with the stage of the menstrual cycle, with implantation and throughout pregnancy, suggesting a measure of endocrine and paracrine control of leukocyte migration to and/or replication in these tissues (Lea and Clark, 1991). Leukocytes make a significant contribution to the stromal compartment of human endometrium, both during the menstrual cycle and in early pregnancy. In the first trimester 30% of cells in the decidual stroma are leukocytes, of which 20–30% are macrophages (Bulmer, 1995; Loke and King, 1995). The major contributors to the leukocyte infiltrate are the CD56 positive large granular lymphocytes (LGL) which account for up to 70% of the leukocyte population in first trimester decidua (Klentzeris *et al.*, 1992; Bulmer, 1995). It is most interesting that the latter cell type accumulate in areas of stromal decidual change, for example near spiral arteries.

The inconsistency in the literature concerning numbers and distributions of macrophages, may reflect the various surface markers utilized to identify macrophage populations and also whether or not the leukocyte is activated. Bulmer *et al.* (1988) described the localization of macrophages in the basal and functional layers throughout the cycle, failed to observe any fluctuations over the cycle, and concluded that macrophage recruitment was not under hormonal control. In contrast, Kamat and Isaacson (1987) have reported a pre-menstrual increase in endometrial macrophage populations. Klentzeris *et al.* (1992) also reported an increase in macrophage numbers in the late secretory phase [luteinizing hormone (LH) + 10 to LH + 13].

The population of phenotypically unique LGL (CD56<sup>+</sup>, CD16<sup>+</sup>, CD3<sup>+</sup>) are reported to increase in the late luteal phase and are localized near glands and spiral arteries (Bulmer *et al.*, 1991). The cell population increases further in the first trimester of pregnancy and numbers decline in the third trimester. Their role is unknown, but a participation in the implantation process has been proposed. It remains to be established whether the increase in this unique subpopulation of LGL is a consequence of in-situ proliferation or due to migration from the circulation (Klentzeris *et al.*, 1992; Marzusch *et al.*, 1993).

Progesterone, however, seems to be essential for the appearance of CD56<sup>+</sup> LGL. Indeed, in ovariectomized women, treatment with both oestrogen and progesterone is necessary for their appearance (Loke and King, 1995). In the absence of pregnancy these unique cells apoptose prior to the influx of neutrophils characteristic of menstruation (Loke and King, 1995). Withdrawal of ovarian progesterone in sheep produced an influx of polymorphonuclear (PMN) leukocytes 24 h after ovariectomy and increased by 48 and 72 h after surgery (Staples

**Table 1.** Age, gestational duration and body mass index of control group and four groups of women (II–V) administered mifepristone (6–36 h) prior to surgical termination of pregnancy. (Values are median, with range in parentheses)

Group	Age (years)	Gestational duration (days)	Body mass index (kg/m <sup>2</sup> )
I (control) (n = 8)	23 (18–38)	58.5 (51–66)	21.6 (19.8–29.7)
II (mifepristone 6 h; n = 7)	20 (18–31)	60.5 (54–67)	25.1 (19.3–27.3)
III (mifepristone 12 h; n = 7)	21 (17–27)	56 (44–67)	25.0 (19.1–28.9)
IV (mifepristone 24 h; n = 7)	22 (20–27)	53 (51–68)	24.8 (21.5–32.6)
V (mifepristone 36 h; n = 7)	20 (17–29)	59 (33–65)	21.2 (18.6–27.2)

*et al.*, 1983). Data concerning neutrophil subpopulations in human endometrium and early decidua are scarce and there are no data on the mechanism of recruitment.

The purpose of this study has been to evaluate the role of progesterone (using a model of progesterone antagonism *in vivo*, that is, administration of the antigestogen mifepristone, RU486) in the recruitment of selected leukocyte subpopulations in early pregnancy decidua.

## Materials and methods

Ethical approval for the study was obtained from Lothian Research Ethics Committee (ref no: 1702/94/6/1). Informed consent was obtained from 36 women undergoing surgical termination of pregnancy by vacuum aspiration between 35 and 63 days amenorrhoea. The women were randomly allocated to five groups (a list was constructed of randomly allocated treatments and patients were assigned to the list as recruited); eight women participated in the control group (group I) and there were seven women in each of four treatment groups: groups II, III, IV and V were administered 200 mg mifepristone (RU486, an antigestogen) at 6, 12, 24 and 36 h respectively before vacuum aspiration. Under general anaesthesia the cervix was dilated using Hegar dilators and decidua tissue away from the implantation area was obtained by careful curettage of the uterine wall prior to vacuum aspiration of the products of conception with a suction catheter. Decidua parietalis (without trophoblast) was subsequently confirmed by examination of haematoxylin and eosin stained sections of each biopsy. Tissue sections were also stained with cytokeratin to confirm the presence or otherwise of trophoblast cells within the decidua tissue. The characteristics of patients in each of the five groups are detailed in Table 1.

## Immunohistochemistry

All biopsies were fixed in 10% neutral buffered formalin at 4°C overnight, rinsed and stored in 70% ethanol and thereafter routinely wax embedded. Sections (5 µm) were cut for immunohistochemical localization of (i) CD45 leukocyte common antigen; (ii) macrophages; (iii) neutrophils; (iv) CD56 positive lymphocytes. Commercially available monoclonal antibodies were used for detection of the leukocyte common antigen CD45 (Dako Laboratories, High Wycombe, UK) macrophages (Dako, CD68, Code M876); neutrophils (DAKO, neutrophil elastase) and CD56 positive lymphocytes (Zymed, San Francisco, CA, USA).

Tissue sections were dewaxed and rehydrated in descending grades

of alcohol. Non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in distilled water for 5 min at room temperature. Pretreatment of the sections was necessary for localization of macrophages and CD56<sup>+</sup> lymphocytes as follows.

## Macrophage immunolocalization

Tissue sections were subjected to an enzyme digestion with 0.1% trypsin in 0.1% calcium chloride at pH 7.8. The digestion was conducted at 37°C for 25 min and subsequently enzyme activity destroyed by washing in tap water.

## CD56<sup>+</sup> lymphocyte immunolocalization

In order to expose the epitope, tissue sections were microwaved at high power in 0.01 M sodium citrate buffer (pH 6.0) for 20 min and then allowed to stand for a further 20 min. Sections were subsequently washed in buffer prior to a non-immune block.

## CD45 leukocyte common antigen and neutrophil elastase

No pretreatment of the tissue was necessary for demonstration of the leukocyte common antigen (CD45) or neutrophil elastase.

All tissue sections were exposed to a non-immune block with normal horse serum performed for 20 min at room temperature. Tissue sections were then incubated with the appropriate primary antibody as follows: (i) leukocyte common antigen (CD45) dilution of antibody 1 in 50 for 60 min at 37°C; (ii) macrophages (CD68) dilution 1 in 50 for 60 min at 37°C; (iii) CD56 dilution 1 in 250 for 60 min at 37°C; (iv) neutrophil elastase dilution 1 in 50 for 60 min at 37°C.

Negative controls were performed by replacing the primary antibody with mouse immunoglobulin at the same concentration as the primary antibody. Sections were labelled with an avidin–biotin–peroxidase detection system (Vector Stain, Vector Laboratories, Peterborough, UK). Tissue sections were incubated for 2–10 min with diaminobenzidine (DAB) solution (DAB Kit, Vector Laboratories) for colour development. Thereafter sections were counter-stained with haematoxylin, dehydrated and cleared in xylene and mounted in Pertex.

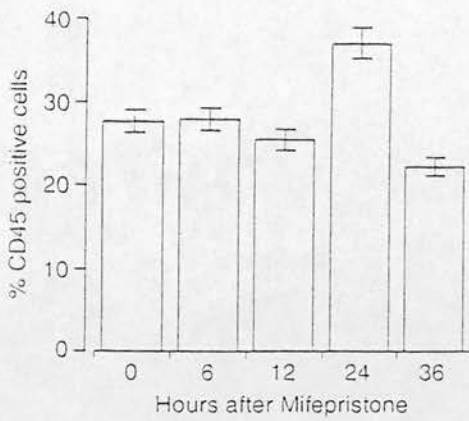
## Image analysis

The immunoreactivity of each leukocyte subpopulation in the stroma of decidual tissue was assessed using image analysis. The system employed an Olympus BH2 microscope (×20 objective) connected to a Macintosh Quadra 700 computer, using Colour Vision software (supplied by Improvision, Coventry, UK).

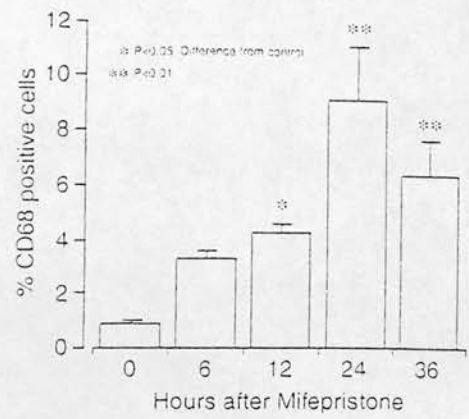
Quantification was performed on the digitized image of systematic randomly selected field from which non-stromal elements (epithelium) had been interactively removed by drawing around the glands. Using colour discrimination software, the total area of positively staining cells (brown reaction product) was measured, and expressed as a ratio to the area of all stromal nuclei (blue haematoxylin). The running mean of the ratio stabilized after measuring 12 fields (Lowrey *et al.*, 1995). In order to represent the amount of positive immunoreactivity in terms of number of cells, the average dimensions of each leukocyte were estimated. Ratios were then converted by a conversion factor such that the data could be expressed as percentages of cells in the stroma.

Samples of decidua were transported to the laboratory in ice-cold RPMI 1640 and then cultured for 24 h in RPMI containing 10% fetal calf serum (Gibco, Paisley, UK) and antibiotics (penicillin/streptomycin and gentamycin). After 24 h culture in 5% CO<sub>2</sub> in air, media were removed, divided into aliquots and stored at –20°C until assayed. Tissue weight was determined after the incubation period. Granulocyte–macrophage colony stimulating factor was assayed using an enzyme-linked immunoassay (ELISA) kit (RandD Systems, Oxford, UK). Interleukin-8 (IL-8) and monocyte chemoattractant





**Figure 1.** CD45 (leukocyte common antigen) positive lymphocytes in control decidua and 6, 12, 24 and 36 h after progesterone withdrawal *in vivo*. No significant change in cell numbers was observed. Values expressed as mean  $\pm$  1 SEM;  $n = 8$  for control subjects and  $n = 7$  subjects for other time points.



**Figure 2.** Appearance of monocytes (CD68<sup>+</sup>) in decidua after administration of mifepristone: \*\* $P < 0.01$ , \* $P < 0.05$  difference from control. Values expressed as mean  $\pm$  1 SEM;  $n = 8$  for control subjects and  $n = 7$  subjects for other time points.

protein-1 (MCP-1) were assayed by ELISA following the method of Ida *et al.* (1992) for IL-8 and Ida *et al.* (1994) for MCP-1. Antisera and standards were the kind gift of Dr M.Naruto. All concentrations of cytokines were corrected for decidual weight.

#### Analysis of data

Analysis of variance (parametric and non-parametric) was used to evaluate whether or not there were significant differences in leukocyte subpopulation number. Regression analysis was also performed.

#### Results

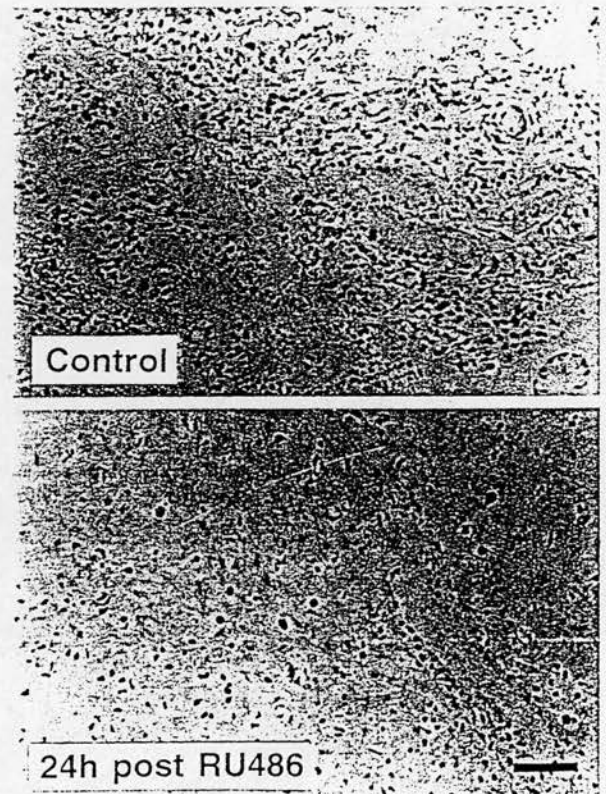
There were no significant changes in the total numbers of leukocytes positive for leukocyte common antigen (LCA, CD45) (Figure 1). However, there was a progressive increase in the number of macrophages (CD68<sup>+</sup>) 12, 24 and 36 h after administration of mifepristone ( $P < 0.01$  and  $< 0.05$ , analysis of variance) (Figure 2). The macrophages were randomly distributed throughout the decidua (Figure 3a and b). There were few neutrophils in decidua and no significant change in numbers was observed after mifepristone (Figure 4). As expected, LGL CD56<sup>+</sup> were abundant in control decidua (Figure 5). There was a slight statistically insignificant decline in numbers after the ingestion of mifepristone. Regression analysis also showed these results to be non-significant.

#### In-vitro studies

Decidual IL-8 concentrations were significantly increased ( $P = 0.019$ ) 6 h after antigestogen administration (Figure 6). Decidual MCP-1 concentrations rose (non-significant) and fell significantly ( $P = 0.029$ ) between 6 and 12 h after mifepristone administration (Figure 7).

#### Discussion

The present study has demonstrated a significant increase in the numbers of monocytes in the decidua parietalis following administration of the antigestogen, mifepristone, *in vivo*. In



**Figure 3.** Photomicrographs of first trimester decidua. (upper) Control biopsy showing only few monocytes (brown); (lower) 24 h after administration of mifepristone. Note increased numbers of tissue monocytes (brown). Scale bar = 50  $\mu$ m.

contrast, no significant changes in CD45 positive (LCA) leukocytes, neutrophils or CD56<sup>+</sup> LGL were observed.

CD45 (LCA) is a pan-leukocyte marker and since the major contributors in the leukocyte infiltrate are the CD56 large granular lymphocytes (up to 70% of leukocytes) we were not surprised at the failure to observe acute changes in the numbers

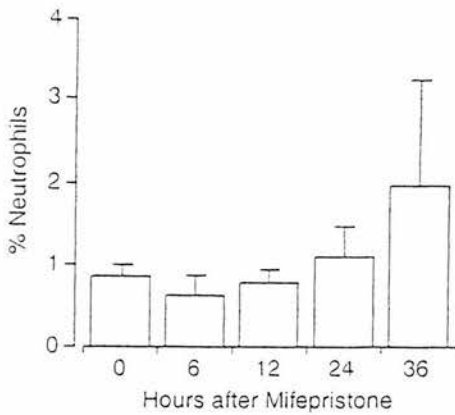


Figure 4. Neutrophil accumulation in control decidua and 6, 12, 24 and 36 h after mifepristone administration. No significant change observed. Values expressed as mean  $\pm$  1 SEM;  $n$  = 8 for control subjects and  $n$  = 7 subjects for other time points.

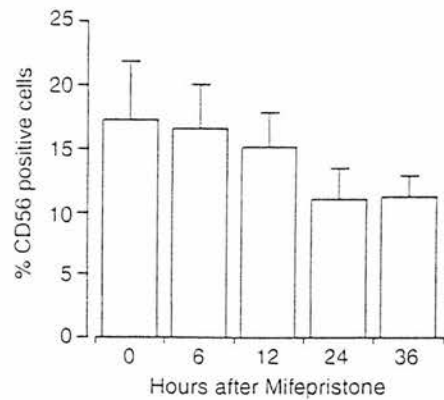


Figure 5. CD56 positive lymphocyte accumulation in control decidua and 6, 12, 24 and 36 h after progesterone antagonism. No significant decrease in numbers was observed. Values expressed as mean  $\pm$  1 SEM;  $n$  = 8 for control subjects and  $n$  = 7 subjects for other time points.

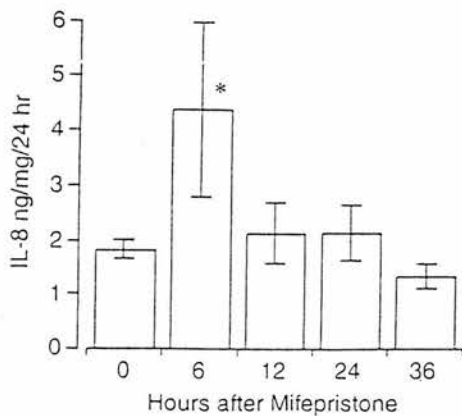


Figure 6. Interleukin (IL)-8 release from cultured explants (ng/mg per 24 h). A significant increase ( $P$  = 0.019) was observed 6 h post-mifepristone administration. Values expressed as mean  $\pm$  1 SEM;  $n$  = 8 for control subjects and  $n$  = 7 subjects for other time points.

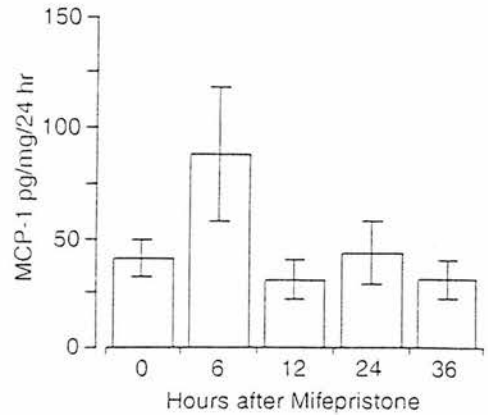


Figure 7. Monocyte chemoattractant protein-1 (MCP-1) release from cultured explants (pg/mg per 24 h). A non-significant rise is observed followed by a significant fall ( $P$  = 0.029) in MCP-1 concentration between 6 and 12 h post-mifepristone administration. Values expressed as mean  $\pm$  1 SEM;  $n$  = 8 for control subjects and  $n$  = 7 subjects for other time points.

of CD45 positive cells after progesterone withdrawal *in vivo*. Indeed our observations are in concordance, in this respect, to the only other published data on CD56+ leukocyte populations in decidua exposed to antigestogen, by Wang *et al.* (1995).

In contrast there was a striking increase in the number of macrophages by 12 h after mifepristone administration. Our findings differ from a previous abstract which reported no change in the number of macrophages or large granulated lymphocytes (CD56<sup>+</sup>) in decidua collected from women at the time of vacuum aspiration 48 h after 200 mg mifepristone (Wang *et al.*, 1995). It is possible that the changes in the number of macrophages which we observed prior to 48 h play an important role in the local cellular responses which lead eventually to abortion.

The local cellular events taking place during decidualization, and indeed menstruation, in the absence of pregnancy, parallel morphological events during an inflammatory process (Finn, 1986; Kelly, 1994). It is likely that steroid dependent events are regulated by a cascade of inflammatory mediators, these being cytokines, growth factors and prostaglandins, which may have their origin in leukocyte sub-populations, i.e. the macrophage and/or non-haematopoietic cells. There are conflicting data on studies of macrophage distribution at the murine maternal-fetal interface. De and Wood (1990) report data to support the role for oestrogen and progesterone in the quantitative and distributional changes observed in uterine macrophage populations. Specifically, ovariectomy in this mouse model caused a significant reduction in macrophage number. More recently, observations by Brandon (1995) have concluded that macrophages are not normally found at the maternal-fetal interface in mice. In the murine model of early embryo loss, Duclos *et al.* (1995) have reported that decidual infiltration with maternal macrophages precedes spontaneous abortion of the early embryo. It is unclear however whether macrophage migration is a result of locally produced tissue signals or whether the migration is a completely random

process. Care, however, must be taken in extrapolating observations in an animal model to mechanisms *in vivo* in the human.

Perhaps one of the earliest observations on the effect of steroids on leukocyte subpopulations is that published in 1928 by Fluhmann. This report noted an increase in macrophage numbers after castrated rabbits were injected with oestrogen. Further support of a role for oestrogen in the regulation of both leukocyte migration, and indeed the production of local inflammatory mediators, is that published recently by Robertson *et al.* (1996). These authors report that oestrogen is the principal regulator of granulocyte-macrophage colony stimulating factor (GmCSF) synthesis by predominantly uterine epithelial cells. Furthermore, administration of mifepristone to ovariectomized non-pregnant mice given oestrogen and progesterone is reported to up-regulate GmCSF synthesis. This important inflammatory mediator may well be responsible for macrophage migration and activation (Wang *et al.*, 1987). There is no evidence as yet which has co-localized an oestrogen receptor on macrophage populations and it is possible therefore that the movement of tissue monocytes is indirectly regulated via steroids. In the context of the present study, antigestogen administration is well known to up-regulate both steroid receptors (Maentausta *et al.*, 1993; Cameron *et al.*, 1996). It is possible therefore that the reported increase in macrophage number in the present study reflects the up-regulation of oestrogen receptor of these decidual tissues *in vivo* after administration of mifepristone (Shi *et al.*, 1993a). In normal decidua oestrogen receptor expression is negligible in both the epithelium and stroma (Shi *et al.*, 1993b).

We have recently observed immunostaining for GmCSF in both control and mifepristone-exposed decidua localized to glandular and surface epithelium. There is a marginal increase in stromal immunoreactivity, especially in the vicinity of blood vessels at 12 h after antigestogen delivery (own unpublished observations). Our present studies (*in vitro*) of GmCSF and other potential local inflammatory mediators showed only a significant increase in decidual IL-8 concentration ( $P = 0.019$ ) at 6 h after mifepristone and a non-significant rise followed by a significant fall in MCP-1 concentration ( $P = 0.029$ ) between 6 and 12 h after mifepristone (Figures 6 and 7).

The present study failed to demonstrate any change in other leukocyte sub-populations. In the absence of pregnancy, CD56<sup>+</sup> LGL apoptose prior to the influx of neutrophils, i.e. at a time when progesterone concentrations are falling prior to menstruation. Thus the survival of CD56<sup>+</sup> cells in the uterus may be progesterone dependent (Loke and King, 1995). Apoptosis of this unique cell population has been observed on days 26 and 27 of the menstrual cycle and in decidua from a failing pregnancy, that is when progesterone concentrations are falling (King *et al.*, 1989; Loke and King, 1995). There is no evidence to date that progesterone causes proliferation of decidual CD56<sup>+</sup> cells and progesterone receptors have not, as yet, been co-localized on this unique cell type (Loke and King, 1995). In this study there was no evidence of changes in CD56<sup>+</sup> cells but this may not preclude apoptotic changes having occurred.

Although it is reported that the proportions of lymphoid populations do not differ between the decidua parietalis and

decidua basalis (Haller *et al.*, 1995), an indirect effect of steroids on leukocytes may occur through the release of cytokines by trophoblast. This may occur via the progesterone receptor recently shown to be present on both villous and extravillous trophoblast populations (Wang *et al.*, 1996). It is possible therefore that effects of mifepristone on leukocyte populations in decidua basalis may differ to that seen in the decidua parietalis. The decidua parietalis, however, covers a vast expanse of fetal membranes in later pregnancy. Both the decidua basalis and parietalis contain cells with morphological features of antigen presenting lymphocytes and lymphocytes capable of immune reactivity with the potential for benefit or harm to the fetal placental unit. Haller *et al.* (1995) have reported that leukocyte patterns in human decidual stroma of basal and parietal decidua are similar. Specifically, macrophages and CD56 positive lymphocytes were the predominant cell populations and evenly distributed.

Thus in summary, we observed a significant increase in tissue monocyte number but not in neutrophils or CD56<sup>+</sup> LGL in the decidua parietalis after progesterone antagonism *in vivo*. Progesterone withdrawal may therefore initiate a local cascade of events involving inflammatory mediators which, in turn, are responsible for the influx of monocytes. Macrophages and neutrophils may be essential in the process of shedding of endometrium or decidua since they are important sources of proteases and collagenases. These cells in turn are potential local sources of immuno-modulatory cytokines.

#### Acknowledgements

The authors wish to acknowledge Sister Catherine Hall for help throughout the study, Dr J.K. Thong for assistance with specimen collection, Mrs Gail Carr and Mrs Sanjukta Chatterjee for technical support and Mrs Vicky Watters for secretarial assistance. Thanks are due to Mr Tom McFetters and Mr Ted Pinner for preparation of the illustrations.

This study was supported by MRC Project Grant No: G9406438PA.

#### References

- Brandon, J.M. (1995) Macrophage distribution in decidual tissue from early implantation to the periparturient period in mice as defined by the macrophage differentiation antigens F4/80, macrophage and the type 3 complement receptor. *J. Reprod. Fertil.*, **103**, 9–16.
- Bulmer, J.N. (1995) Immune cells in decidua. In Kurpisz, M. and Fernandez, N. (eds), *Immunology of Human Reproduction*. BIOS Scientific Publishers Ltd, Oxford, UK, pp. 313–334.
- Bulmer, J.N., Lunny, D.P. and Hagin, S.V. (1988) Immunohistochemical characterisation of stromal leukocytes in non-pregnant human endometrium. *Am. J. Reprod. Immunol. Microbiol.*, **17**, 83–92.
- Bulmer, J.N., Longfellow, M. and Ritson, A. (1991) Leukocytes and resident blood cells in endometrium. *Ann. N. Y. Acad. Sci.*, **622**, 57–68.
- Cameron, S.T., Critchley, H.O.D., Buckley, C.H. *et al.* (1996) The effects of post-ovulatory administration of onapristone on the development of a secretory endometrium. *Hum. Reprod.*, **11**, 40–49.
- De, M., and Wood, G.W. (1990) Influence of oestrogen and progesterone on macrophage distribution in the mouse uterus. *J. Endocrinol.*, **126**, 417–424.
- Duclos, A.J., Haddad, E.K. and Baines, M.G. (1995) Presence of activated macrophages in a murine model of early embryo loss. *Am. J. Reprod. Immunol.*, **33**, 354–366.
- Finn, C.A. (1986) Implantation, menstruation and inflammation. *Biol. Rev.*, **61**, 313–328.
- Fluhmann, C.H. (1928) The reticulo endothelial cells of the uterus: an experimental study. *Am. J. Obstet. Gynecol.*, **15**, 783–796.



- Haller, H., Tedesco, F., Rukavina, D. *et al.* (1995). Decidual-trophoblast interactions: decidual lymphoid cell populations in basal and parietal decidua. *J. Reprod. Immunol.*, **28**, 165-171.
- Ida, N., Sakurai, S., Hosoi, K. *et al.* (1992) A highly sensitive enzyme-linked immunosorbent assay for the measurement of interleukin-8 in biological fluids. *J. Immunol. Methods*, **156**, 27-38.
- Ida, N., Sakurai, S. and Kawano, G. (1994) Detection of monocyte chemotactic and activating factor (MCAF) in normal blood and urine using sensitive ELISA. *Cytokine*, **6**, 32-39.
- Kamat, B.R. and Isaacson, P.G. (1987) The immunocytochemical distribution of leukocytic sub populations in human endometrium. *Am. J. Pathol.*, **127**, 66-73.
- Kelly, R.W. (1994) Pregnancy maintenance and parturition: the role of prostaglandin in manipulating the immune and inflammatory response. *Endocr. Rev.*, **15**, 684-706.
- King, A., Wellings, V., Gardner, L. *et al.* (1989) Immunocytochemical characterisation of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. *Hum. Immunol.*, **24**, 195-205.
- Klentzeris, L.D., Bulmer, J.N., Warren, A. *et al.* (1992) Endometrial lymphoid tissue in the timed endometrial biopsy. Morphometric and immunohistochemical aspects. *Am. J. Obstet. Gynecol.*, **167**, 667-674.
- Lea, R.G. and Clark, D. (1991) Macrophages and migratory cells in endometrium relevant to implantation. *Baillieres Clin. Obstet. Gynaecol.*, **5**, 25-29.
- Loke, Y.W. and King, A. (1995) Uterine mucosal leukocyte. In Loke, Y.W. and King, A. (eds), *Human Implantation. Cell Biology and Immunology*. Cambridge University Press, Cambridge.
- Lowrey, J.A., Williams, A.R.W., Howie, S.E.M. *et al.* (1995) A novel image analysis based technique for a quantitation of lymphoid cells in endometrial stroma. *J. Pathol.*, **176**, 23A.
- Maentausta, O., Svalander, P., Gemzell-Danielsson, K. *et al.* (1993) The effects of an antiprogesterin, mifepristone and an anti-estrogen, tamoxifen, on endometrial 17 $\beta$  hydroxysteroid dehydrogenase and progesterin and estrogen receptors during the luteal phase of the menstrual cycle: an immunohistochemical study. *J. Clin. Endocrinol. Metab.*, **77**, 913-918.
- Marzusch, K., Ruck, P., Geiselhart, A. *et al.* (1993) Distribution of cell adhesion molecules on CD56<sup>+</sup>, CD3<sup>+</sup>, CD16<sup>+</sup> large granular lymphocytes and endothelial cells in first trimester human decidua. *Hum. Reprod.*, **8**, 1203-1208.
- Robertson, S.A., Mayrhofer, G. and Seamark, R.F. (1996) Ovarian steroid hormones regulate granulocyte-macrophage colony-stimulating factor synthesis by uterine epithelial cells in the mouse. *Biol. Reprod.*, **54**, 183-196.
- Shi, W., Wang, J.D., Fu, Y. *et al.* (1993a) The effect of RU486 on progesterone receptor in villous and extravillous trophoblast. *Hum. Reprod.*, **8**, 953-958.
- Shi, W., Wang, J.D., Fu, Y. *et al.* (1993b) Estrogen and progesterone receptors in human decidua after RU486 treatment. *Fertil. Steril.*, **60**, 69-74.
- Staples, L.D., Heap, R.B., Wooding, F.B.P. *et al.* (1983) Migration of leucocytes into the uterus after removal of ovarian progesterone during early pregnancy in the sheep. *Placenta*, **4**, 339-350.
- Wang, J.M., Colella, S., Allavena, P. *et al.* (1987) Chemotactic activity of human recombinant granulocyte-macrophage colony-stimulating factor. *Immunology*, **60**, 439-444.
- Wang, J.D., Fu, Y., Zhu, J.B., *et al.* (1995) Lymphocyte population in human decidua after RU486. *Biol. Reprod.*, **52**, 184.
- Wang, J.D., Zhu, J.B., Fu, Y. *et al.* (1996) Progesterone receptor immunoreactivity at the maternofetal interface of first trimester pregnancy: a study of the trophoblast population. *Hum. Reprod.*, **11**, 413-419.

Received on May 16, 1996; accepted on July 31, 1996

## Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation

Rebecca L. Jones<sup>1,3</sup>, Rodney W. Kelly<sup>2</sup> and Hilary O.D. Critchley<sup>1</sup>

<sup>1</sup>Department of Obstetrics and Gynaecology, University of Edinburgh and <sup>2</sup>Medical Research Council Reproductive Biology Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh, EH3 9EW, UK

<sup>3</sup>To whom correspondence should be addressed

The endometrium contains a resident population of leukocytes, the number and subtype of which vary throughout the menstrual cycle and in early pregnancy. Factors controlling these fluctuations are unknown, but a combination of proliferation *in situ* and migration from the vasculature has been proposed. Locally acting inflammatory mediators, including specific chemokines and prostaglandins, have been implicated in these processes. Interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) are potent chemoattractants and activators for neutrophils and monocytes respectively. Locally acting prostaglandins modulate vascular permeability, and a synergistic action of prostaglandin E (PGE) with IL-8 has been described. In the present study IL-8, MCP-1 and cyclooxygenase-2 (COX-2), the inducible isoform of prostaglandin synthase, were all localized in the endometrium by immunohistochemistry throughout the menstrual cycle and in early pregnancy. All three inflammatory mediators were localized to the perivascular cells of blood vessels in endometrium and decidua, and additional immunoreactivity for COX-2 was identified in the glandular epithelium. The intensity of immunostaining was reduced in the periovulatory, early and mid-secretory phases and significantly increased premenstrually. These results further support the hypothesis that there is a premenstrual migration of leukocytes into the endometrium mediated by chemokines.

**Key words:** cyclooxygenase-2/endometrium/interleukin-8/leukocytes/monocyte chemoattractant protein-1

### Introduction

The human endometrium undergoes rapid proliferation and differentiation under the control of the ovarian steroid hormones oestrogen and progesterone. Associated with the progesterone mediated decidualization in the mid-late secretory phase is the accumulation of leukocytes in the endometrial stroma (Bulmer *et al.*, 1991a; Starkey *et al.*, 1991). These comprise macrophages and the uterine specific large granular lymphocytes (LGLs), and both subtypes persist in early pregnant decidua (Loke and King, 1995). In the absence of

pregnancy, a distinct influx of neutrophils is observed in the immediate premenstrual phase (Poropatich *et al.*, 1987).

The exact function of the endometrial population of leukocytes is unknown, although a role in the regulation of implantation and placentation and also in the initiation of menstruation has been suggested (Bulmer *et al.*, 1991b; King and Loke, 1991). The factors controlling their mode of appearance and fluctuation throughout the menstrual cycle are similarly uncertain. The cyclical pattern of leukocyte presence is suggestive of steroidal control, but the recent demonstration that they do not possess either oestrogen or progesterone receptors implies that this regulation is exerted indirectly (King *et al.*, 1996). Co-localization studies have revealed that the stromal leukocytes express the cell proliferation marker Ki67 (Pace *et al.*, 1989; King *et al.*, 1991), indicating that their increase in numbers is due at least in part to *in-situ* proliferation. It is likely, however, that the migration of leukocytes from the peripheral circulation also contributes to leukocyte accumulation.

Migration is a multistep process involving both the simultaneous expression of leukocyte adhesion molecules and their corresponding receptors on endothelial cells, and additionally the production of specific chemotactic agents (Springer, 1990; Schall and Bacon, 1994). A family of cytokines exhibiting chemotactic activity for specific leukocytes are the chemokines (for reviews see Matsushima *et al.*, 1992; Baggiolini *et al.*, 1994). Interleukin-8 (IL-8), a potent chemoattractant and activator of neutrophils, has been detected in the human cervix (Barclay *et al.*, 1993), endometrium (Arici *et al.*, 1993; Critchley *et al.*, 1994), chorio-decidua (Dudley *et al.*, 1993) and the placenta (Saito *et al.*, 1994). Increased production of IL-8 in the cervix coincides with neutrophil influx prior to ripening at term (Kelly *et al.*, 1992; El Maradny *et al.*, 1996). IL-8 has been localized to the perivascular cells of blood vessels in non-pregnant and pregnant endometrium (Critchley *et al.*, 1994), where it could participate in recruitment of neutrophils and additionally in the process of angiogenesis (Koch *et al.*, 1992).

A suppression of IL-8 production by the endometrium (Kelly *et al.*, 1994) and cervix (Ito *et al.*, 1994) *in vitro* by progesterone has been demonstrated, acting either through the glucocorticoid response element in the promoter region of the gene or directly through progesterone receptors present within the cell. If a similar downregulation by progesterone occurs *in vivo*, the withdrawal of progesterone premenstrually would remove the inhibitory effect, resulting in an increased production of IL-8 and accompanying neutrophil influx.

A closely related chemokine is monocyte chemoattractant protein-1 (MCP-1). MCP-1 displays no chemotactic activity

for neutrophils; instead it is a potent attractant and activator for macrophages, T cells (Cai *et al.*, 1995; Roth *et al.*, 1995), basophils, mast cells (Feliciani *et al.*, 1995) and also natural killer cells (Allavena *et al.*, 1994). It is positively regulated by pro-inflammatory stimuli, such as interleukin-1 (IL-1) and tumour necrosis factor (TNF)- $\alpha$ , as is IL-8 (Baggiolini *et al.*, 1994), but is additionally induced by platelet derived growth factor (PDGF) in fibroblasts (Yoshimura and Leonard, 1990). This growth factor has no effect on IL-8 production, indicating that regulatory differences in the gene expression of these chemokines exist. Little is known about the downregulation of MCP-1, with the exception of an inhibition by glucocorticoids in certain cell lines (Brach *et al.*, 1992; Shyy *et al.*, 1995). MCP-1 production by endometrial cells has recently been described (Akoum *et al.*, 1996), but a role for MCP-1 in leukocyte recruitment to the normal endometrium has not been investigated.

The infiltrate of leukocytes into tissue is frequently accompanied by the leakage of plasma producing stromal oedema. Permeability of blood vessels is modulated by vasoactive substances including prostaglandins (PGs). Prostaglandin E (PGE), a potent vasodilator, and the antagonist vasoconstrictor prostaglandin F<sub>2</sub> $\alpha$  (PGF<sub>2</sub> $\alpha$ ), have been implicated in the modulation of blood vessel tone prior to and during menses (Baird *et al.*, 1996). A role in the initiation of menstruation is further reinforced by the observed synergism between PGE and IL-8 in the infiltration of neutrophils from the peripheral circulation (Colditz, 1990). Prostaglandins are synthesized by the cyclooxygenase (COX) enzyme, which exists in two differentially regulated isoforms (Goppelt-Streube, 1995). COX-1 is constitutively expressed in most cell types, whilst COX-2 is induced transiently in response to inflammatory stimuli (Zweifel *et al.*, 1995). Decidual cells have been demonstrated to produce COX-2 in response to IL-1 $\beta$ , and this induction can be inhibited by dexamethasone and progesterone (Ishihara *et al.*, 1995). This finding reinforces the observation that prostaglandin release from proliferative and secretory endometrium is decreased by progesterone (Abel and Baird, 1980; Kelly and Smith, 1987).

The present study has investigated the localization and temporal expression of the chemokines IL-8 and MCP-1 and also COX-2 in the endometrium throughout the menstrual cycle and in early pregnancy.

## Materials and methods

### Tissue collection

Endometrial biopsies ( $n = 44$ ) were collected by Pipelle suction curette (Laboratoire CCD, Paris, France) from women undergoing minor gynaecological procedures (Table I). All women reported normal regular menstrual cycles (25–35 days) and had not received any form of hormonal treatment for the preceding 3 months. Biopsies were dated from the patient's last menstrual period (LMP) and tissue sections were examined to ensure that histological appearances were consistent with the date of LMP. In addition, first trimester decidua ( $n = 17$ ) collected away from the implantation site was biopsied by curettage prior to suction termination of pregnancy. Decidua parietalis, without trophoblast invasion, was confirmed by cytokeratin immunostaining. Written informed consent was obtained prior to tissue

Table I. Stages of menstrual cycle at time of biopsy collection

Stage of menstrual cycle	Number of biopsies
Menstrual (days 1–4)	6
Early proliferative (days 5–7)	3
Mid-proliferative (days 8–10)	4
Late proliferative (days 11–13)	5
Peri-ovulatory (day 14)	5
Early secretory (days 15–18)	10
Mid-secretory (days 19–24)	9
Late secretory (days 25 to menstruation)	7
Decidua (8–10 weeks)	24

collection and ethical approval was received from Lothian Research Ethics Committee. Tissue samples were immediately placed in OCT embedding medium (Tissue-Tek, Miles Inc., Elkhart, USA) and snap frozen in iso-pentane precooled with dry ice. Additionally, a sample was immersion fixed in 10% neutral buffered formalin (NBF) overnight at 4°C, prior to paraffin embedding. Subsequently, 5  $\mu$ m sections were cut from both the frozen and paraffin blocks for immunohistochemical investigation.

### Immunohistochemistry

Immunohistochemical protocols for the detection of MCP-1 and COX-2 were optimized to determine the correct conditions for maximal specific staining. Methodology for the immunolocalization of the chemokine IL-8 has previously been reported (Crichtley *et al.*, 1994).

### MCP-1

Immediately prior to staining, frozen sections were lightly fixed in 10% NBF for 10 min at room temperature. Sections were then washed in 0.1 M phosphate-buffered saline (PBS) pH 7.4–7.6. Endogenous peroxidase activity was quenched by immersion of the slides in 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; BDH Laboratory Supplies, Poole, UK) in distilled water for 5 min at room temperature. Following 10 min washing in PBS, diluted non-immune goat serum (Vectastain Elite PK-6101; Vector Laboratories, Peterborough, UK) was applied to each slide and the slides were incubated for 20 min in a humidified chamber. The excess serum was carefully blotted off and 50  $\mu$ l of MCP-1 antibody (raised in rabbits using a chemically synthesized 77 amino acid MCP-1 as the antigen) was applied at a dilution of 1:400 in PBS. Coverslips were placed over the tissue sections to minimize antibody evaporation and the slides were incubated overnight (17 h  $\pm$  1) at 4°C. Following primary antibody binding, an avidin–biotin peroxidase detection system was utilized, involving the sequential application of biotinylated goat anti-rabbit IgG (Vectastain Elite PK-6101, Vector Laboratories) and a complex of avidin and biotin with horseradish peroxidase (ABC Vectastain 4002; Vector Laboratories). The positive binding was then identified by the application of the peroxidase substrate 3, 3'-diaminobenzidine (DAB, Vector SK-4100; Vector Laboratories) which produces a brown stain representing the presence of MCP-1. Sections were then washed in distilled water and lightly counterstained with Harris's haematoxylin (Pioneer Research Chemicals Ltd, Colchester, UK), a non-specific purple nuclear stain. Following washing in tap water, the sections were dehydrated in ascending grades of ethanol and mounted from xylene with Pertex mounting medium (Cellpath plc, Hemel Hempstead, UK).

### IL-8

A similar protocol was used for IL-8 immunolocalization, with the exception of an incubation period of 60 min at 37°C with the primary



rabbit polyclonal antibody raised against the IL-8 peptide, at a dilution of 1:500.

### COX-2

Immunostaining for COX-2 was conducted on paraffin sections. These were dewaxed in HistoClear (National Diagnostics, Atlanta, Georgia, USA), and rehydrated through descending grades of ethanol. Prior to endogenous peroxide blocking, a microwave antigen retrieval technique was utilized, whereby the sections were heated in sodium citrate buffer (pH 6.0) for 10 min at high power followed by a 20 min incubation in the oven. A similar protocol as for MCP-1 was then continued, with the exception of 60 min incubation periods with the primary antibody (PG27, rabbit polyclonal; Oxford Biomedical, Biogenesis, Poole, UK) diluted 1:250 at 37°C, the secondary antibody and the tertiary ABC Elite (Vectastain Elite PK-6101; Vector Laboratories), both at room temperature.

### Controls

Frozen tonsil tissue sections were included as a positive control for both chemokine immunostaining procedures, and were treated in an identical fashion. COX-2 immunoreactivity was confirmed by the inclusion of third trimester fetal membranes in each staining run. To assess the specificity of the immunoreactivity, serial sections of representative slides were also included and either non-immune rabbit IgG or primary antibody preabsorbed with the appropriate synthetic peptide (IL-8 or MCP-1) (100 µg/ml) was substituted for the primary antibody.

### Scoring and analysis of immunostaining

A semi-quantitative scoring system was employed for assessment of intensity and localization of immunoreactivity in the entire tissue section, where a score of 0 indicates an absence of immunoreactivity, 1 faint immunoreactivity, 2 strong immunoreactivity and 3 very intense immunoreactivity. Scoring was conducted blind to the stage of the cycle, randomly by one observer. Analysis of variance (ANOVA) with Fisher's PLSD to assign significance was used to evaluate whether or not there were significant differences in the expression of the mediators.

### Results

The local mediators, MCP-1, IL-8 and COX-2, were detected by immunohistochemistry in samples of endometrium from non-pregnant and pregnant women (see Figure 1). Immunostaining was primarily present in the perivascular cells around all blood vessel types in the endometrial stroma for MCP-1 and IL-8, although weaker immunoreactivity was identified in the glandular epithelium. Additional staining was detected for MCP-1 in the cytoplasm of a subpopulation of stromal cells in occasional aggregates in some decidual biopsies. Intense immunoreactivity for COX-2 was detected in the epithelial glands and perivascular cells. In the menstrual phase and in some of the decidual sections examined, a degree of stromal staining was observed.

Tonsil sections included as a positive control for MCP-1 and IL-8 immunostaining also exhibited perivascular immunostaining. Immunoreactivity for COX-2 in third trimester fetal membranes was detected primarily in the amniotic epithelium and chorion laeve trophoblast, with slight immunostaining in the attached decidual cells, in agreement with published data (Gibb and Sun, 1996). Antibody preabsorbed with the

appropriate peptide, or non-immune rabbit IgG applied to serial tissue sections to act as a negative control, resulted in an absence of immunoreactivity.

### MCP-1

MCP-1 immunoreactivity was detected in a cyclical pattern during the menstrual cycle (Figure 2). Throughout the proliferative phase MCP-1 immunostaining was relatively intense. A distinct reduction in staining intensity was observed in the periovulatory phase and levels remained low in the early and mid-secretory stages. In the premenstrual or late secretory phase, however, immunostaining increased significantly ( $P < 0.05$ ) compared with the periovulatory stage. First trimester decidua exhibited reduced immunoreactivity with respect to premenstrual levels, comparable with mid-secretory immunostaining.

### IL-8

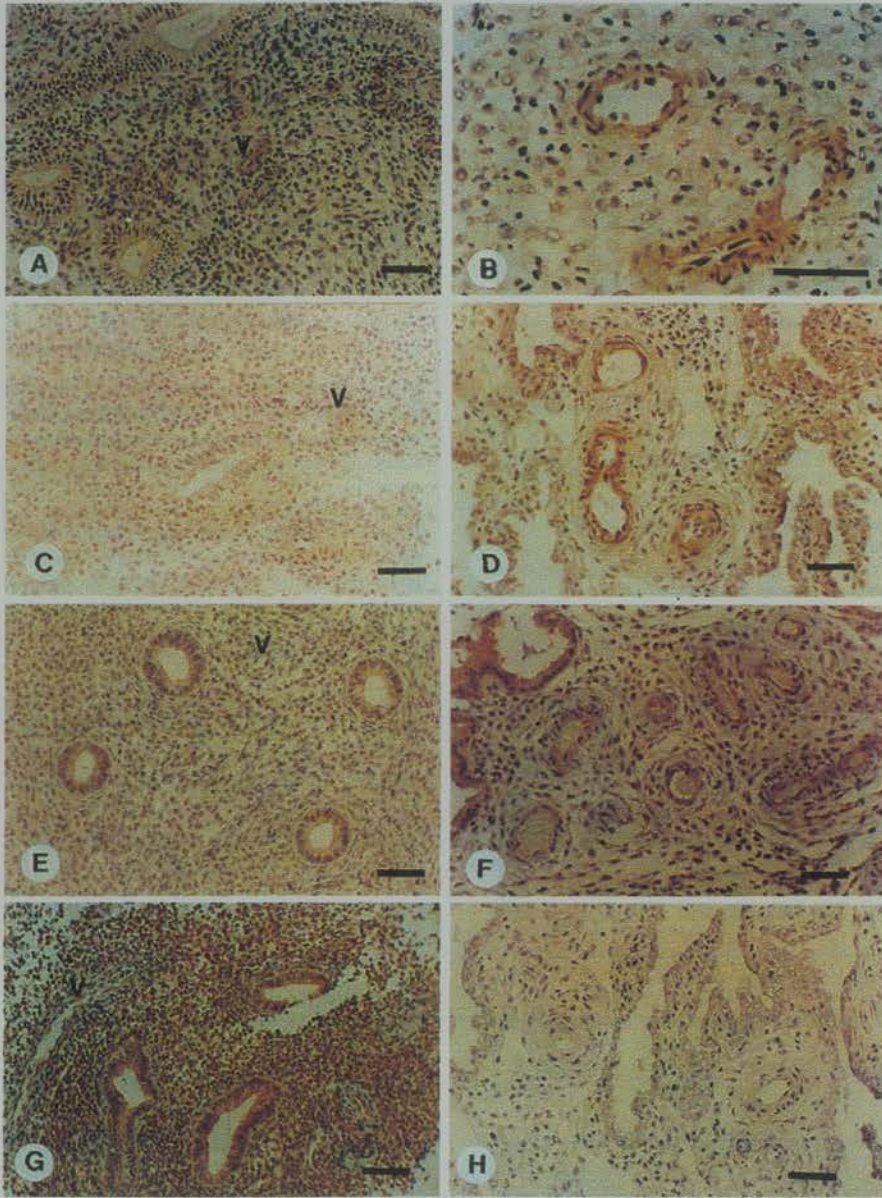
The intensity of IL-8 immunostaining also varied throughout the menstrual cycle (Figure 3). Low levels were detected in the perivascular cells in the menstrual and proliferative phases of the menstrual cycle. As for MCP-1, there was a clear reduction in staining intensity in the periovulatory phase, although this did not reach significance. Immunostaining levels increased slightly in the early and mid-secretory phase, but a highly significant increase ( $P < 0.001$ ) in staining intensity was apparent in the late secretory phase in comparison with the ovulatory and early secretory phase. In the decidua, lower levels of IL-8 immunoreactivity compared to MCP-1 immunostaining levels were observed. The average immunoreactivity was significantly ( $P < 0.01$ ) lower than that observed premenstrually and was consistent with the expression of IL-8 in the mid-secretory phase.

### COX-2

COX-2 immunoreactivity was observed at all stages of the menstrual cycle (Figure 4). Both glands and vessels exhibited intense immunostaining in the menstrual phase. In the proliferative stage, levels of immunoreactivity decreased slightly and lower levels still were detected in the ovulatory phase. This pattern continued through the early and mid-secretory phases and a significant increase in COX-2 immunoreactivity was observed in the late secretory phase (glandular  $P < 0.01$ , vessels  $P < 0.05$ ). Glandular immunoreactivity remained high in early decidua whilst perivascular staining appeared to be reduced (not significant).

### Discussion

MCP-1, IL-8 and COX-2 were localized in the endometrium and decidua by immunohistochemistry. The localization of these chemotactic agents to the perivascular cells in both pregnant and non-pregnant endometrium is consistent with a putative role in the recruitment of specific leukocytes from the vasculature. Elevated levels of chemokine immunoreactivity were detected in the premenstrual phase of the menstrual cycle. These findings support the hypothesis that migration of leukocytes from the peripheral circulation contributes to the



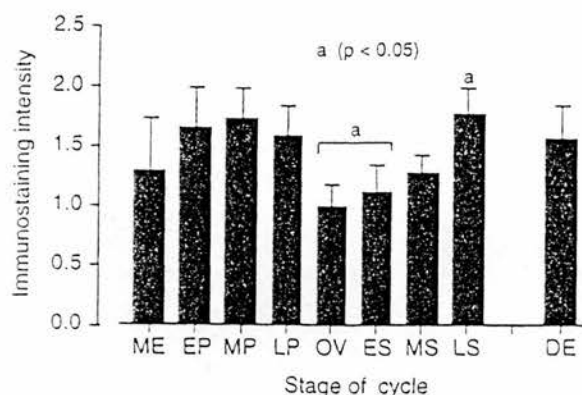
**Figure 1.** Immunohistochemical localization of IL-8, MCP-1 and COX-2 in human endometrium. (A) Periovulatory endometrium immunostained for IL-8. Faint immunoreactivity is apparent around small vessels. (B) Increased intensity of perivascular IL-8 immunoreactivity in late secretory endometrium. (C) MCP-1 immunoreactivity in periovulatory endometrium, demonstrating an absence of immunostaining. (D) First trimester decidua exhibits a marked increase in MCP-1 immunostaining intensity, in the perivascular cells of all vessels. (E) COX-2 immunoreactivity in periovulatory endometrium. Faint immunostaining is observed in the glandular epithelium and vessels. (F) Premenstrual endometrium exhibits intense immunostaining for COX-2 in both epithelial glands and perivascular cells. (G) Additional stromal immunoreactivity is observed in menstrual endometrium. (H) Negative control for MCP-1, decidual section treated with antibody preabsorbed with MCP-1 peptide. Scale bars, 50  $\mu$ m, V = vessel.

significant leukocyte accumulation observed premenstrually. Furthermore, the co-localization of COX-2 in the perivascular cells reinforces the proposed interaction of prostaglandins and chemokines in the local inflammatory response.

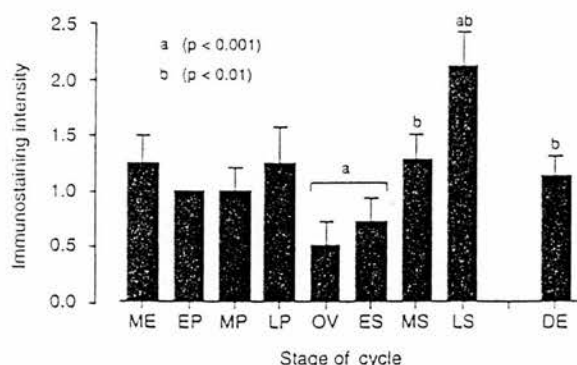
The exact cell types in the perivascular region which exhibit immunoreactivity have not been characterized. Endothelial cells, fibroblasts and smooth muscle cells are present in this

location, and all have been demonstrated to be capable of chemokine production (see reviews by Matsushima and Oppenheim, 1989; Baggiolini *et al.*, 1994). Additional immunoreactivity was identified in a subpopulation of stromal cells in some decidual sections. These observations may represent a population of leukocytes with the potential for chemokine production.



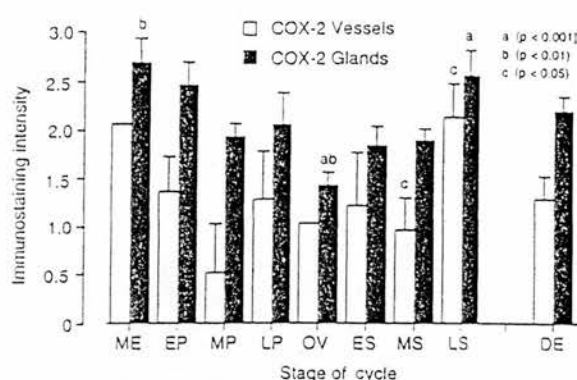


**Figure 2.** MCP-1 immunoreactivity throughout the menstrual cycle and in early pregnancy. A significant increase in immunoreactivity levels is apparent in the premenstrual phase with respect to the ovulatory and early secretory phases ( $P < 0.05$ ). Significant differences between groups are denoted by matching letters. Y-axis error bars represent the standard error of the mean (SEM). ME, menstrual; EP, early proliferative; MP, mid-proliferative; LP, late proliferative; OV, ovulatory; ES, early secretory; MS, mid-secretory; LS, late secretory; DE, decidua.



**Figure 3.** IL-8 immunoreactivity throughout the menstrual cycle and in early pregnancy. A highly significant increase in immunostaining occurs in the premenstrual phase compared to levels in the ovulatory and early secretory phases ( $P < 0.001$ ) and the mid-secretory phase ( $P < 0.01$ ). In early pregnancy, significantly lower levels of immunoreactivity were detected than premenstrually ( $P < 0.01$ ). Significant differences between groups are denoted by matching letters. Y-axis error bars represent the standard error of the mean (SEM). Abbreviations as in Figure 2.

Examination of tissues obtained throughout the menstrual cycle and in early pregnancy revealed a cyclical pattern in the intensity of immunoreactivity for all three mediators. Most noticeable was the distinct reduction in staining intensity around the time of ovulation, extending into the early secretory phase. This coincides with the initiation of progesterone production from the corpus luteum and peak oestrogen production. At this stage the endometrium is most responsive to the effects of oestrogen and progesterone due to maximal steroid receptor expression (Snijders *et al.*, 1992). Furthermore, the perivascular cells identified as the site of chemokine production strongly express progesterone receptors throughout the cycle (Perrot-Applanat *et al.*, 1988; Bouchard *et al.*, 1991). Experimental evidence has demonstrated that MCP-1 secretion by



**Figure 4.** COX-2 immunoreactivity throughout the menstrual cycle and in early pregnancy. Glandular immunostaining is represented by filled bars and perivascular immunostaining by white bars. Glandular immunoreactivity was significantly elevated in the menstrual ( $P < 0.01$ ) and premenstrual phases ( $P < 0.001$ ) compared to the ovulatory phase. Perivascular immunostaining followed a similar pattern, with a significant increase in the premenstrual phase ( $P < 0.05$ ) with respect to the early secretory phase. Significant differences between groups are denoted by matching letters. Y-axis error bars represent the standard error of the mean (SEM). Abbreviations as in Figure 2.

monocytes and fibroblasts in culture is inhibited by oestrogen (Kovacs *et al.*, 1993, 1996). A similar regulation may occur *in vivo*, although the levels of MCP-1 immunoreactivity detected during the proliferative phase would not support this. The levels of chemokine and COX-2 immunoreactivity detected remain low through the early and mid-secretory phases, until the late secretory phase, when a significant elevation in expression is apparent. At this stage in the non-pregnant cycle, regression of the corpus luteum results in a drop in progesterone concentrations. If pregnancy occurs, progesterone values are maintained and this corresponds to a lower level of immunoreactivity for all three mediators. Thus it appears that the staining intensity for all three mediators is reduced in the presence of progesterone and increased with declining progesterone concentrations premenstrually. These observations are consistent with a downregulation of the chemokines and COX-2 by progesterone, such as has been demonstrated *in vitro* (Ito *et al.*, 1994; Kelly *et al.*, 1995; Ishihara *et al.*, 1995). This would represent an anti-inflammatory action of progesterone, in the suppression of a local inflammatory response at the time when fertilization and implantation may occur. The method by which progesterone may achieve this downregulation is as yet unknown, but this immunohistochemical evidence of a co-localisation of responsive cells and progesterone receptors suggests a receptor-mediated indirect effect of progesterone in leukocyte migration.

Supporting evidence for a downregulation of chemokine expression by progesterone is the increase in the numbers of macrophages in first trimester decidua, following the pharmacological withdrawal of progesterone by administration of the antigestogen mifepristone (Critchley *et al.*, 1996). This observation may be related to an increase in chemokine expression.

The phenotypically unique natural killer-like LG (CD56<sup>+</sup>CD16<sup>+</sup>) are the predominant leukocyte subtype in the



late secretory endometrium and first trimester decidua. Their origin is uncertain, but it has been suggested that a precursor form may be recruited from peripheral blood into the endometrial stroma, where a subsequent activation/differentiation occurs (King *et al.*, 1991). A putative precursor cell type are the CD56<sup>+</sup>CD16<sup>+</sup> agranular cells which comprise ~1% of the circulating NK cells (Lanier *et al.*, 1986). Chemotactic activity for NK cells has been attributed to MCP-1 (Allavena *et al.*, 1994). Further investigation is necessary to determine whether MCP-1 may play a role in the accumulation of LGLs in the mid-late secretory phase and in the first half of pregnancy. IL-8 has also been implicated in the chemotaxis of lymphocytes (Larsen *et al.*, 1989), and this has been linked to the LGL infiltrate in the endometrium (Casey and MacDonald, 1993). However, it has recently been proposed that only the C-C chemokines (including MCP-1) and not the C-X-C subfamily, which includes IL-8, possess chemotactic ability for T lymphocytes (Roth *et al.*, 1995).

The co-localization of IL-8 and COX-2 in the endometrium reinforces the synergistic action of PGE in the IL-8 stimulated recruitment of neutrophils (Colditz, 1990). Furthermore, as MCP-1 and IL-8 are closely related chemokines, it is possible that PGE may also have an enhancing effect on monocyte infiltration. Prostaglandin activity is also regulated by the locally produced metabolizing enzyme prostaglandin dehydrogenase (PGDH). A positive regulation by progesterone has been demonstrated by the reduced immunoreactivity for PGDH following antigestogen administration in early pregnancy (Cheng *et al.*, 1993b). Also observed was increased immunoreactivity for PGE<sub>2</sub> around the small blood vessels in the endometrium (Cheng *et al.*, 1993a). Thus progesterone may affect local prostaglandin concentrations by modulating the enzymes responsible for both their production and metabolism.

The localization of COX-2 primarily to the glandular epithelium supports the in-vitro evidence that glands are the major site of prostaglandin synthesis in the endometrium (Lumsden *et al.*, 1984; Smith and Kelly, 1988). Furthermore, whilst the endometrium retains the ability to synthesize prostaglandins in response to stimuli throughout the menstrual cycle, higher levels of prostaglandins are released from isolated glands in the proliferative than in the secretory phase and early pregnancy (Smith and Kelly, 1988). This is consistent with a stimulation of prostaglandin production by oestrogen (Abel and Baird, 1980), potentially through cyclooxygenase upregulation (Jun *et al.*, 1996), and a downregulation by progesterone.

The present study has therefore identified and immunolocalized the inflammatory mediators MCP-1, IL-8 and COX-2 to the perivascular cells in endometrium and decidua. A cyclical pattern of immunoreactivity has been observed, with maximum expression of MCP-1, IL-8 and COX-2 occurring premenstrually and coinciding with the large increase in leukocytes in the endometrial stroma at this time. These observations support a role for steroid-regulated chemokines in the mechanisms determining leukocyte accumulation in endometrium and decidua.

#### Acknowledgements

The authors would like to thank clinicians and theatre staff at the Royal Infirmary Edinburgh, especially Dr Andrew Paterson and Dr

Richard Anderson, for their assistance in the collection of endometrium and decidua. Technical assistance from Miss Teresa Drury is also acknowledged.

#### References

- Abel, M.H. and Baird, D.T. (1980) The effect of 17 $\beta$  estradiol and progesterone on prostaglandin production by human endometrium maintained in organ culture. *Endocrinology*, **106**, 1599–1606.
- Akoun, A., Lemay, A., McColl, S. *et al.* (1996) Elevated concentration and biologic activity of monocyte chemoattractant protein-1 in the peritoneal fluid of patients with endometriosis. *Fertil. Steril.*, **66**, 17–23.
- Allavena, P., Bianchi, G., Zhou, D. *et al.* (1994) Induction of natural-killer-cell migration by monocyte chemoattractant protein-1, protein-2 and protein-3. *Eur. J. Immunol.*, **24**, 3233–3236.
- Arici, A., Head, J., MacDonald, P. *et al.* (1993) Regulation of interleukin-8 gene expression in endometrial cells in culture. *Mol. Cell. Endocrinol.*, **94**, 195–204.
- Baggiolini, M., Dewald, B. and Moser, B. (1994) Interleukin-8 and related chemotactic cytokines – CXC and CC chemokines. *Adv. Immunol.*, **55**, 97–179.
- Baird, D.T., Cameron, S.T., Critchley, H.O.D. *et al.* (1996) Prostaglandins and menstruation. *Eur. J. Obstet. Gynaecol. Reprod. Biol.*, **70**, 15–17.
- Barclay, C.G., Brennand, J.E., Kelly, R.W. *et al.* (1993) Interleukin-8 production by the human cervix. *Am. J. Obstet. Gynecol.*, **169**, 625–632.
- Bouchard, P., Marraoui, J., Massai, M.R. *et al.* (1991) Immunocytochemical localisation of oestradiol and progesterone receptors in human endometrium: a tool to assess endometrial maturation. *Bailliere's Clin. Obstet. Gynaecol.*, **5**, 107–115.
- Brach, M., Gruss, H., Riedel, D. *et al.* (1992) Effects of antiinflammatory agents on synthesis of MCP-1/JE transcripts by human blood monocytes. *Mol. Pharmacol.*, **42**, 63.
- Bulmer, J.N., Longfellow, M. and Ritson, A. (1991a) Leukocytes and resident blood cells in endometrium. *Ann. N.Y. Acad. Sci.*, **622**, 57–68.
- Bulmer, J.N., Morrison, L., Longfellow, M. *et al.* (1991b) Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies. *Hum. Reprod.*, **6**, 791–798.
- Cai, J.-P., Hudson, S. and Chin, Y.-H. (1995) Stimulation of human T cell trans-dermal microvascular endothelial cell migration by MCP-1 – abstract. *J. Invest. Dermatol.*, **104**, 558.
- Casey, M.L. and MacDonald, P.C. (1993) Cytokines in the human placenta, fetal membranes, uterine decidua and amniotic fluid. In Rice, G.E. and Brennecke, S.P. (eds), *Molecular Aspects of Placental and Fetal Membrane Autocoids*. CRC Press, Boca Raton.
- Cheng, L., Kelly, R.W., Thong, K.J. *et al.* (1993a) The effect of (RU486) on the immunohistochemical distribution of prostaglandin E and its metabolite in decidua and chorionic tissue in early pregnancy. *J. Clin. Endocrinol. Metab.*, **77**, 873–877.
- Cheng, L., Kelly, R.W., Thong, K.J. *et al.* (1993b) The effects of mifepristone (RU486) on prostaglandin dehydrogenase in decidua and chorionic tissue in early pregnancy. *Hum. Reprod.*, **8**, 705–709.
- Colditz, I.G. (1990) Effect of exogenous prostaglandin E<sub>2</sub> and actinomycin D on plasma leakage induced by neutrophil activating peptide-1/interleukin-8. *Immunol. Cell. Biol.*, **68**, 397–403.
- Critchley, H.O.D., Kelly, R.W. and Kooy, J. (1994) Perivascular expression of chemokine interleukin-8 in human endometrium. *Hum. Reprod.*, **9**, 1406–1409.
- Critchley, H.O.D., Kelly, R.W., Lea, R.G. *et al.* (1996) Sex steroid regulation of leucocyte traffic in human decidua. *Hum. Reprod.*, **11**, 2257–2262.
- Dudley, D.J., Trautman, M.S. and Mitchell, M.D. (1993) Inflammatory mediators regulate IL-8 production by cultured gestational tissues: evidence for a cytokine network at the chorio-decidual interface. *J. Clin. Endocrinol. Metab.*, **76**, 404–410.
- El Maradny, E., Kanayama, N., Halim, A. *et al.* (1996) Biochemical changes in the cervical tissue of rabbit induced by interleukin-8, interleukin-1 $\beta$ , dehydroepiandrosterone sulphate and prostaglandin E<sub>2</sub>: a comparative study. *Hum. Reprod.*, **11**, 1099–1104.
- Feliciani, C., Theoharides, T., Mammarella, S. *et al.* (1995) Activation of mast cells by MCP-1. *J. Invest. Dermatol.*, **104**, 685.
- Gibb, W. and Sun, M. (1996) Localisation of prostaglandin-H synthase type 2 (PGHS-2) protein and mRNA in term human fetal membranes and decidua. *J. Endocrinol.*, **150**, 497–503.
- Goppelt-Streube, M. (1995) Regulation of prostaglandin endoperoxide synthase

- (cyclooxygenase) isoenzyme expression. *Prostaglandins Leukot. Essent. Fatty Acids*, 52, 213–222.
- Ishihara, O., Matsuoka, K., Kinoshita, K. *et al.* (1995) Interleukin-1 $\beta$ -stimulated PGE<sub>2</sub> production from early first trimester human decidual cells is inhibited by dexamethasone and progesterone. *Prostaglandins*, 49, 15–26.
- Ito, A., Imada, K., Sato, T. *et al.* (1994) Suppression of interleukin-8 production by progesterone in rabbit uterine cervix. *Biochem. J.*, 301, 183–186.
- Jin, S.S., Chen, Z., Sherman, T.S., *et al.* (1996) Estrogen up-regulates cyclooxygenase gene expression in fetal pulmonary artery endothelial cells. *Pediatr. Res.*, 39, 164.
- Kelly, R.W. and Smith, S.K. (1987) Glucocorticoids do not share with progesterone the potent inhibitory action of prostaglandin synthesis in human proliferative phase endometrium. *Prostaglandins*, 33, 919–929.
- Kelly, R.W., Leask, R. and Calder, A.A. (1992) Chorionic production of IL-8 and mechanism of parturition. *Lancet*, 339, 776–777.
- Kelly, R.W., Illingworth, P., Baldie, G. *et al.* (1994) Progesterone control of interleukin-8 production in endometrium and chorio-decidual cells underlines the role of the neutrophil in menstruation and parturition. *Hum. Reprod.*, 9, 253–258.
- King, A. and Loke, Y.W. (1991) On the nature and function of human uterine granular lymphocytes. *Immunol. Today*, 12, 432–435.
- King, A., Balendran, N., Wooding, P. *et al.* (1991) CD3<sup>+</sup> leukocytes present in the human uterus during early pregnancy: phenotypic and morphologic characterisation of the CD56<sup>+</sup> population. *Dev. Immunol.*, 1, 169–190.
- King, A., Gardner, L. and Loke, Y.W. (1996) Evaluation of oestrogen and progesterone receptor expression in uterine mucosal lymphocytes. *Hum. Reprod.*, 11, 1079–1082.
- Koch, A.E., Polverini, P.J., Kunkel, S.L. *et al.* (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science*, 258, 1798–1801.
- Kovacs, E.J., DonCarlos, L.L. and Frazier-Jensen, M.R. (1993) Estrogen inhibits JE/MCP-1 expression in macrophages – abstract. *J. Immunol.*, 150, A84.
- Kovacs, E.J., Faunce, D.E., Rammerquinn, D.S. *et al.* (1996) Estrogen regulation of JE/MCP-1 messenger RNA expression in fibroblasts. *J. Leukoc. Biol.*, 59, 562–568.
- Lanier, L.L., Le, A.M., Civin, C.I. *et al.* (1986) The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J. Immunol.*, 136, 4480–4486.
- Larsen, C.G., Andeson, A.O., Appella, E. *et al.* (1989) The neutrophil-activating peptide (NAP-1) is also chemotactic for T lymphocytes. *Science*, 243, 1464–1466.
- Loke, Y.W. and King, A. (1995) Uterine mucosal leucocytes. In Loke, Y.W. and King, A. (eds), *Human Implantation, Cell Biology and Immunology*. Cambridge University Press, Cambridge, pp. 102–129.
- Lumsden, M.A., Brown, A. and Baird, D.T. (1984) Prostaglandin production from homogenates of separated glandular epithelium and stroma from human endometrium. *Prostaglandins*, 28, 485–496.
- Matsushima, K. and Oppenheim, J.J. (1989) Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL-1 and TNF. *Cytokine*, 1, 2–13.
- Matsushima, K., Baldwin, E.T. and Mukaida, N. (1992) Interleukin-8 and MCAF: novel leukocyte recruitment and activating cytokines. In Kishimoto, T. (ed.), *Interleukins: Molecular Biology and Immunology*. Karger, Basel, pp. 236–265.
- Pace, D., Morrison, L. and Bulmer, J.N. (1989) Proliferative activity in endometrial stromal granulocytes through the menstrual cycle and early pregnancy. *J. Clin. Pathol.*, 42, 35–39.
- Perrot-Applanat, M., Groyer-Picard, M.T., Garcia, E. *et al.* (1988) Immunocytochemical demonstration of oestrogen and progesterone receptors in muscle cells of uterine arteries in rabbits and humans. *Endocrinology*, 123, 1511–1519.
- Poropatich, C., Rojas, M. and Silverberg, S.G. (1987) Polymorphonuclear leukocytes in the endometrium during the normal menstrual cycle. *Int. J. Gynecol. Pathol.*, 6, 230–234.
- Roth, S.J., Woldemar Carr, M. and Springer, T.A. (1995) C-C chemokines, but not the C-X-C chemokines interleukin-8 and interferon- $\gamma$  inducible protein-10, stimulate transendothelial chemotaxis of T lymphocytes. *Eur. J. Immunol.*, 25, 3482–3488.
- Saito, S., Kasahara, T., Sakakura, S. *et al.* (1994) Detection and localisation of interleukin-8 mRNA and protein in human placenta and decidual tissues. *J. Reprod. Immunol.*, 27, 161–172.
- Schall, T. and Bacon, K. (1994) Chemokines, leukocyte trafficking, and inflammation. *Curr. Opin. Immunol.*, 6, 865–873.
- Shyy, Y.-J., Li, Y.-S., Lin, M.-C. *et al.* (1995) Dexamethasone inhibits the shear stress induced MCP-1 gene expression – abstract. *FASEB J.*, 9, A322.
- Smith, S.K. and Kelly, R.W. (1988) The release of PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> from separated cells of human endometrium and decidua. *Prostaglandins Leukot. Essent. Fatty Acids*, 33, 91–96.
- Snijders, M.P., de Goeij, A.F., Debets-Te Baerts, M.J.C. *et al.* (1992) Immunocytochemical analysis of oestrogen receptors and progesterone receptors in the human uterus throughout the menstrual cycle and after the menopause. *J. Reprod. Fertil.*, 94, 363–371.
- Springer, T.A. (1990) Adhesion receptors of the immune system. *Nature*, 346, 425–434.
- Starkey, P., Croser, L. and Rees, M. (1991) Variation during the menstrual cycle of immune cell populations in human endometrium. *Eur. J. Obstet. Gynaecol. Reprod. Biol.*, 39, 203–207.
- Yoshimura, T. and Leonard, E.J. (1990) Secretion by human fibroblasts of monocyte chemoattractant protein-1, the product of the gene JE. *J. Immunol.*, 144, 2377–2383.
- Zweifel, B.S., Colburn, S.M., Orgberg, R.L. *et al.* (1995) The role of cyclooxygenase-2 in inflammation. *FASEB J.*, 9, A110.

Received on December 9, 1996; accepted on March 17, 1997

## Localization and Temporal Expression of Prolactin Receptor in Human Endometrium

R. L. JONES, H. O. D. CRITCHLEY, J. BROOKS, H. N. JABBOUR, AND  
A. S. McNEILLY

Department of Obstetrics and Gynecology, University of Edinburgh, and the Medical Research Council Reproductive Biology Unit, Center for Reproductive Biology (J.B., H.N.J., A.S.M.), Edinburgh, Scotland EH3 9EW

### ABSTRACT

Extrapituitary PRL is synthesized by the decidualized endometrial stromal cells from the mid to late secretory phase in the nonpregnant cycle and throughout pregnancy. The function of PRL in the uterus is unknown, but the temporal expression indicates a role in implantation and placentation. PRL is a powerful immunoregulatory agent, and thus, a role in modulating endometrial leukocytes may be envisaged. To investigate the site of action of PRL, immunohistochemistry was conducted to localize the PRL receptor (PRL-R). In addition, ribonucleic acid was extracted and reverse transcriptase-PCR for PRL-R was conducted. PRL-R protein was immunolocalized to the

glandular epithelium and a subset of stromal cells from the mid to late secretory phase of the menstrual cycle and in early decidua. PRL-R transcripts were also detected from the late secretory phase and first trimester decidua. These findings indicate that the receptor is expressed in a temporal pattern similar to that of PRL. PRL-R expression in the glandular epithelium is consistent with a role in regulating glandular activity. Furthermore, immunoreactivity for PRL-R in a subset of stromal cells may be evidence for paracrine interactions between decidualized cells or an immunoregulatory role for PRL. (*J Clin Endocrinol Metab* 83: 258–262, 1998)

EXTRAPITUITARY PRL is produced and secreted by the decidua during pregnancy (1). The decidualized stromal cells have been conclusively shown to be the sole site of synthesis (2). Furthermore, experimental studies have recognized that PRL is produced by the nonpregnant endometrium from the mid to late luteal phase until menses (3). This corresponds to the initiation of decidualization during which the stromal cells become pseudodecidualized (2), and *in vitro* decidualization by stimulation of estrogen-primed proliferative endometrial cells with progesterone results in PRL production (4). Indeed, PRL expression is now commonly used as a marker for functional decidualization. Although pituitary and endometrial PRL are identical in structure and function, expression is differentially regulated, which has been attributed to the presence of a distinct promoter region located ~6 kilobases upstream of the pituitary promoter (5, 6).

The significance of PRL expression in the endometrium is uncertain. It is believed that PRL primarily acts as an autocrine or paracrine, rather than endocrine, factor. A similar mechanism has been reported in immune cells, where PRL has a profound immunomodulatory effect (7). The majority of leukocytes ubiquitously express the PRL receptor (PRL-R) (8–11). Stimulated T cells secrete PRL (10), and *in vitro* PRL enhances the proliferation of leukocytes previously stimulated with mitogenic factors, including interleukin-2 and T and B cell mitogens (12). Furthermore, this proliferation is inhibited by exposure to anti-PRL antibodies (13). *In vivo*

studies indicate an immunosuppressive action, such as the reduced immune response in lactating or PRL-treated castrated rats (14, 15).

The temporal expression of PRL in the endometrium suggests a role in the preparation for implantation and subsequent placentation. Decidualization is essential for implantation to occur, and the specialized immune environment within the decidua plays a major role in achieving a successful pregnancy. Coincidental with decidualization is the accumulation of leukocytes in the endometrial stroma, the majority of which are the uterine-specific large granular lymphocytes and macrophages (16, 17). These persist into early pregnancy and are believed to have a role in regulating implantation and placentation (18). PRL may be acting as an immunomodulatory agent in the endometrium by stimulation of either leukocyte proliferation or differentiation.

Although the production of PRL by the endometrium has been thoroughly investigated, its site of action in the endometrium has not been fully elucidated. PRL-R have been detected in the amniochorion, where PRL is believed to have an osmoregulatory effect on amniotic fluid volume (19), and additionally in third trimester decidua (20, 21). The exact cellular localization and temporal expression in the nonpregnant and early pregnant endometrium have not been clearly examined. The present study, by means of immunohistochemistry and reverse transcriptase-PCR (RT-PCR), investigates PRL-R expression in the human endometrium at different stages of the menstrual cycle and early pregnancy.

### Materials and Methods

#### Tissue collection

Normal endometrial tissue (menstrual, n = 3; proliferative, n = 7; ovulatory, n = 3; early secretory, n = 3; mid secretory, n = 5; late

Received April 3, 1997. Revision received July 11, 1997. Rerevision received September 24, 1997. Accepted September 30, 1997.

Address all correspondence and requests for reprints to: Dr. Rebecca L. Jones, Department of Obstetrics and Gynecology, Center for Reproductive Biology, 37 Chalmers Street, Edinburgh, Scotland EH3 9EW.



secretory,  $n = 6$ ) was collected from fertile women with regular menstrual cycles (25–35 days) who were undergoing minor gynecological procedures. Decidua parietalis ( $n = 8$ ) was obtained by curettage before surgical termination of first trimester pregnancies. Written informed consent was received before tissue collection, and ethical approval was received from Lothian research ethics committee. Tissue samples were fixed by immersion in 10% neutral buffered formalin overnight at 4°C and stored in 70% ethanol before routine paraffin embedding. In addition, a sample was snap-frozen in isopentane precooled with dry ice and stored at  $-70^{\circ}\text{C}$ . Ribonucleic acid (RNA) was subsequently extracted using Ultraspec RNA Isolation System (Biogenesis, Poole, UK). The RNA yield was estimated by measuring absorbance at 260 nm by spectrophotometry, and purity was assessed by the ratio of absorbance at 260:280 nm.

### Immunohistochemistry

The conditions for optimal specific immunoreactivity were determined. Paraffin sections (5  $\mu\text{m}$ ) were dewaxed in Histoclear (National Diagnostics, Atlanta, GA) and rehydrated through descending grades of ethanol to distilled water. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ , BDH Laboratory Supplies, Poole, UK) in distilled  $\text{H}_2\text{O}$  for 5 min. Nonspecific binding was reduced by a 20 minute incubation with nonimmune goat serum (Vectastain Elite, Vector Laboratories, Peterborough, UK) in a humidified chamber at room temperature, after which the primary antibody (rabbit polyclonal raised against a rat peptide sequence common to the short and long forms of the receptor, supplied by Dr. P. Ingleton, University of Sheffield, Sheffield, UK) (22) was applied at a dilution of 1:50. The slides were incubated overnight (17  $\pm$  1 h) at 4°C. Antibody binding was detected by the sequential application of biotinylated goat antirabbit IgG and an avidin-biotin-peroxidase complex (ABC Vectastain, Vector Laboratories, Peterborough, UK). The substrate 3,3'-diaminobenzidine (Vector Laboratories) was then used to visualize positive immunoreactivity. Finally, sections were counterstained with Harris's hematoxylin (Pioneer Research Chemicals, Colchester, UK), dehydrated in ethanol, and mounted from xylene (BDH Laboratory Supplies).

Human term fetal membranes were included as a positive control for the PRL-R immunohistochemistry (21). A matching concentration of nonimmunized rabbit IgG was substituted for the primary antibody to exclude the possibility of nonspecific binding.

### RT-PCR

Total RNA extracted from tissue collected at all stages throughout the menstrual cycle (proliferative,  $n = 12$ ; secretory,  $n = 12$ ) and in early pregnancy ( $n = 6$ ) was subjected to RT-PCR for PRL-R.

### Glyceraldehyde phosphate dehydrogenase (GAP-DH)

To ensure equivalent loading of viable RNA for the PRL-R RT-PCR, the expression level of the housekeeping gene GAP-DH was monitored by RT-PCR. Oligonucleotide primers for GAP-DH (sense, 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3'; antisense, 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3') were designed to produce a 1103-bp product. A sample of 400 ng total RNA was reverse transcribed to complementary DNA (cDNA) using avian myeloblastosis virus RT (Promega, Southampton, UK) and subsequently amplified by PCR using AmpliTaq DNA polymerase (Perkin-Elmer, Beaconsfield, UK) for 28 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, followed by a final extension for 10 min at 72°C. Analysis of products by electrophoresis enabled assessment of viability of RNA, and bands of comparable intensity indicated comparable RNA loading into the RT-PCR.

### PRL-R

Oligonucleotide primers complementary to the extracellular domain of the PRL-R were used (sense, 5'-GCA GAT GGA GGA CTT CCT ACC AAT TA-3'; antisense, 5'-GCA GGT CAC CAT GCT ATA GCC CTT-3'), flanking bp 154–794, to amplify by RT-PCR a 650-bp product. The primers chosen spanned intronic sequences to ensure against the possibility of genomic DNA contamination. Firstly, gene-specific cDNA for PRL-R was generated by the reverse transcription of 400 ng total RNA

from stocks identical to those used for GAP-DH, using the antisense oligonucleotide primer at 42°C for 45 min. The total volume of RT products was then amplified by PCR for 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, followed by a 10-min final extension at 72°C. Sheep pituitary RNA was included as a positive control for the RT-PCR (23), and the omission of template RNA served as a negative control. The products were analyzed by comparison with a mol wt marker (PCR Marker, Sigma, Poole, UK) on a 1% agarose gel containing ethidium bromide.

The identity of the RT-PCR product was confirmed by subcloning into expression vector pGEM-T Easy Vector (Promega) and sequenced using the dideoxy chain termination method.

### Southern hybridization

The identity of the amplified PRL-R products were further confirmed by Southern hybridization (24). Representative RT-PCR products, amplified from RNA extracted at different stages throughout the menstrual cycle, were selected for analysis. The products were separated by electrophoresis on a 1% agarose gel and blotted overnight onto a nylon membrane by capillary transfer. Hybridization was conducted overnight at 42°C with a 562-bp cDNA, labeled with [ $\alpha$ - $^{32}\text{P}$ ]deoxy-CTP by random priming, encoding the conserved extracellular domain (82% homology with human PRL-R cDNA) of the red deer PRL-R (between bp 96 and 657) (25). Posthybridization washes in SSC (standard saline citrate) and SDS were conducted with increasing stringency, and the membrane was thereafter exposed to autoradiographic film for 3 weeks.

### Results

Specific immunoreactivity for PRL-R was detected in endometrium and decidua by immunohistochemistry (Fig. 1). In the proliferative and early secretory phases of the non-pregnant cycle, immunostaining was weak or absent in the stroma, with minimal immunoreactivity visible in the luminal region of the glandular epithelium. From the mid to late secretory phase and in early pregnancy, intense immunoreactivity was apparent in both the glands and a subpopulation of stromal cells. In addition, positive immunostaining was identified in the blood vessels of some of the decidualized endometrial tissues. Fetal membranes included to act as a positive control exhibited positive immunoreactivity in the amniotic epithelium, chorion-laeve trophoblast, and decidual layer as previously reported (21). Substitution of the primary antibody with rabbit IgG resulted in an absence of staining in fetal membranes and endometrial and decidual sections.

After PRL-R RT-PCR, a single band of 650 bp was detected by gel electrophoresis, corresponding to the region of the PRL-R gene amplified. Confirmation of its identity was obtained by Southern hybridization (Fig. 2), and sequence analysis verified that the amplicon encoded human PRL-R. A band representing PRL-R was detected in the tissue biopsied during the menstrual phase (days 1–4). During the proliferative and early secretory stages, the signal was either weak or absent. From the mid to late secretory phase, however, a strong signal was detected, with increasing intensity in samples obtained from early pregnancy (Fig. 3). A GAP-DH signal of comparable intensity (1103 bp) was obtained from all samples, indicating that equivalent amounts of RNA were loaded into the RT-PCR (Fig. 3). Furthermore, the presence of a GAP-DH signal in the absence of a PRL-R signal indicated that this was not due to an absence of viable RNA, but to the inability to detect PRL-R messenger RNA in the RNA sample. Overall, although this method is not totally quan-

FIG. 1. PRL-R immunolocalization in human endometrium. Minimal immunoreactivity is observed in the proliferative phase (A) and early secretory phase (B) endometrium. In the mid to late secretory phase (C), intense immunostaining is present in the epithelial glands and a subpopulation of stromal cells. This pattern persists in first trimester decidua (D), and at higher power (E) the decidualized stromal cells clearly exhibit strong immunoreactivity. Immunostaining is also apparent in the decidual vasculature (v). The negative control (F), in which the primary antibody was substituted with nonimmune rabbit IgG, results in an absence of staining. G, Glandular epithelium. Scale bars = 50  $\mu$ m.

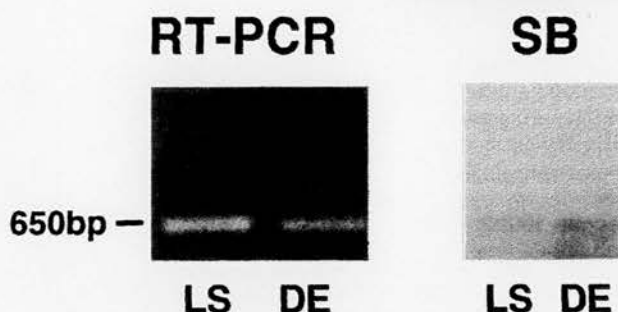
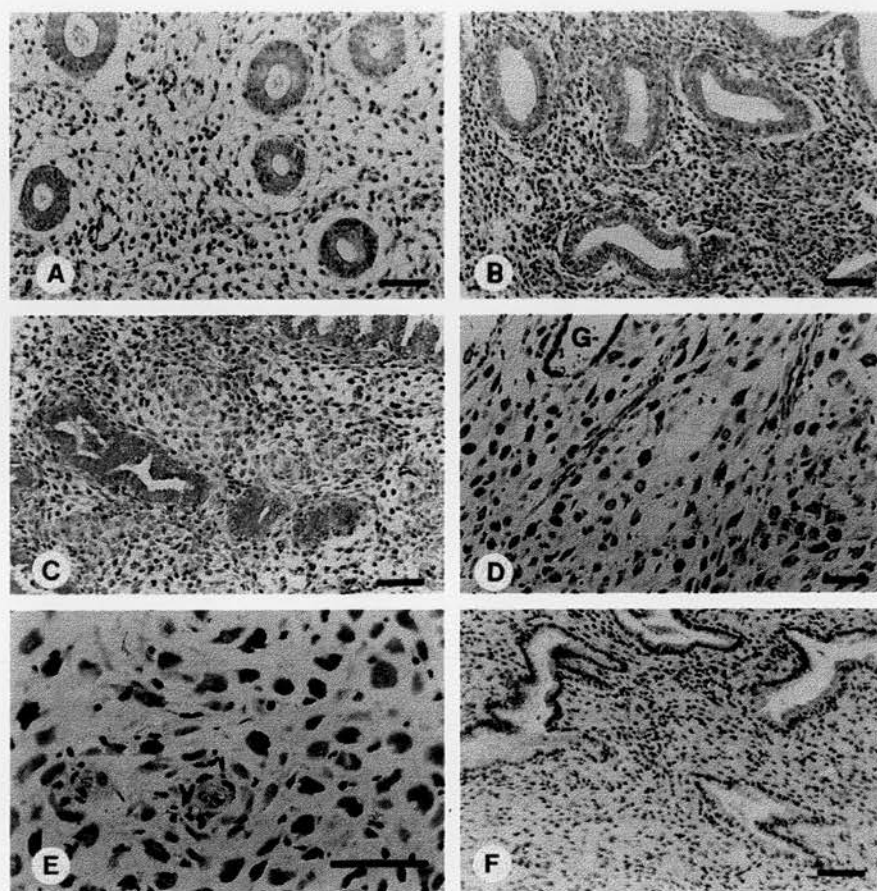


FIG. 2. RT-PCR for PRL-R generated a 650-bp amplified product, as identified by comparison with a mol wt marker. The identity of the amplicon was confirmed by Southern blotting (SB) and hybridization with a 562-bp cDNA probe raised against the conserved extracellular domain. LS, Late secretory; DE, decidua.

titative, these data suggest that PRL-R expression is up-regulated in the mid to late secretory phase and continues in early pregnancy.

### Discussion

This study confirms the expression of the PRL-R in endometrium and decidua. Immunohistochemical localization revealed that the PRL-R is strongly expressed by the glandular

epithelium and stromal cells in pseudodecidualized and decidualized endometrium. Immunostaining was minimal or absent in proliferative and early secretory phase endometrium. This temporal pattern of expression was confirmed by the amplification of PRL-R messenger RNA by RT-PCR, with the detection of a strong signal from the mid to late secretory phase of the nonpregnant cycle and in early pregnancy. Thus, the temporal expression of the receptor appears to mirror that of PRL by the decidualized endometrium.

It seems likely that progesterone has a role in the regulation of PRL-R expression (26). It is unlikely to be a direct regulation, however, as is the case for PRL, whose production is stimulated and maintained by the progesterone-mediated decidualization of the endometrium (27, 28). Regulation via decidua-derived factors (29) or even PRL itself may be envisaged (30–32). An indirect regulation by progesterone is firmly supported by the absence of progesterone receptors in the glandular epithelium when PRL-Rs are expressed.

The detection of strong immunoreactivity for the receptor in the epithelial glands suggests a paracrine action for PRL. During the early secretory phase, the endometrial glands become tortuous and actively produce uterine secretions, which are vital for preparing the endometrium for implantation (33). During the decidualization of the uterine stroma and continuing into pregnancy, the glandular epithelium

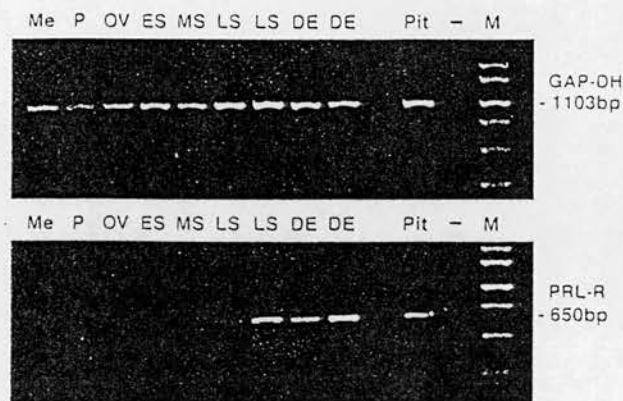


FIG. 3. RNA extracted from tissues from each stage of the menstrual cycle and early pregnancy was subjected to RT-PCR to determine the temporal expression of PRL-R. The upper panel shows representative RNA samples amplified for GAP-DH (amplicon size, 1103 bp), indicating the comparable loading of viable RNA into the PRL-R RT-PCR. The lower panel shows the same RNA samples amplified for PRL-R (amplicon size, 650 bp). No signal was obtained in the menstrual (Me), proliferative (P), ovulatory (OV), or early secretory (ES) phases. From the mid and late secretory phases (MS/LS), a signal was achieved, which increased in intensity in early pregnancy (DE, decidua). Pituitary (Pit) RNA was included as a positive control, yielding a band of identical size. No signal was observed for the negative control (-).

becomes atrophic, and glandular secretory activity ceases. PRL has been reported to possess osmoregulatory activity, and the large amounts of PRL detectable in amniotic fluid act as a regulator of amniotic fluid volume. This was demonstrated by a link between defective PRL-R expression in amniotic epithelium and the incidence of polyhydramnios (19). The presence of PRL-R in the glandular epithelium during decidualization implicates a similar role for PRL, *i.e.* in the down-regulation of glandular activity.

Additional immunoreactivity was observed in a subset of stromal cells. The identity of these stromal cells is unknown, although the majority display morphological characteristics of decidualized stromal cells. This further supports the likelihood of the existence of an autocrine and/or paracrine interaction between PRL and its receptor. Further colocalization studies are required to determine whether the PRL-producing cells concurrently express the receptor. Positive immunostaining for the receptor was detected in the vasculature of some decidualized endometrial samples examined. The perivascular cells frequently exhibited immunostaining as expected, as this is the site of initiation of decidualization in the endometrium. Occasional staining was also observed that appeared to be endothelial in nature. A 16-kDa N-terminal fragment of human PRL has been noted to be a potent inhibitor of angiogenesis (34), and a high affinity binding site has been previously identified on endothelial cells (35).

PRL has important immunoregulatory functions in the peripheral immune system, including leukocyte proliferation, differentiation, and function. An important feature of the premenstrual endometrium and first trimester decidua is the accumulation of leukocytes within the decidualized stroma. These are believed to be essential for successful implantation and placentation (16), but the mechanism respon-

sible for the increase in the secretory phase is not fully understood. Of particular interest are the uterus-specific large granular lymphocytes. It has been speculated that PRL may have a role in their proliferation and/or differentiation, especially after the discovery that these cells, although clearly under the influence of progesterone (36), do not express sex steroid receptors (37). The effect of PRL treatment on these decidual lymphocytes has been examined (36, 37), but both studies failed to observe a resultant proliferation or differentiation. It is not unlikely, however, that PRL may have an effect on decidual leukocytes, acting indirectly through autocrine/paracrine factors produced by neighboring cells.

It is probable that the migration of specific leukocytes from the peripheral circulation also contributes to the increase in leukocyte numbers. This is supported by the elevated levels of the chemokines interleukin-8 and monocyte chemoattractant protein-1 in the endometrium during leukocyte accumulation (38). PRL may be involved in enhancing this recruitment, as recent data reveal that the production of monocyte chemoattractant protein-1 is stimulated by PRL in ovarian tissue (39). A number of roles for PRL in the immunomodulation of the uterine leukocytes can thus be visualized, with a potential for augmenting lymphocyte migration, differentiation, and proliferation. It remains to be determined whether the endometrial leukocytes express the PRL-R.

In third trimester amniochorion, PRL has been implicated in the enhancement of matrix metalloproteinase activity (21, 40). During menses and placentation in early pregnancy, a dramatic reorganization of extracellular matrix must occur to allow endometrial shedding and trophoblast invasion, respectively. These processes invariably involve the action of matrix metalloproteinases, and it is possible that PRL may participate as one of the factors capable of altering the activities of these enzymes in the endometrium.

In summary, this study clearly demonstrates the expression of PRL-R in decidualized nonpregnant and pregnant endometria, with a temporal pattern similar to that of PRL. Immunohistochemical studies have isolated the glands and a subset of stromal cells as sites of action for PRL. PRL has multiple functions throughout the body, including roles in growth, differentiation, and immune response, and it is therefore difficult to isolate a single function for PRL in the uterus. It is, however, likely that PRL plays an important role in immunoregulation and additionally in the decidualization of both stromal and glandular cells of the endometrium.

#### Acknowledgments

We extend our thanks to Miss Joanne McVerry and Miss Sheila Boddy for their technical assistance, and to Mr. Tom McFetters and Mr. Edward Pinner for all their help with the figures. Additionally, we acknowledge the help of Dr. Rodney Kelly in the RT-PCR studies.

#### References

1. Ben-Jonathon N, Mershon JL, Allen DL, Steinmetz RW. 1996 Extrapituitary prolactin: distribution, regulation, functions and clinical aspects. *Endocr Rev.* 17:639-669.
2. Wu W-X, Brooks J, Glasier AF, McNeilly AS. 1995 The relationship between decidualization and prolactin mRNA and production at different stages of human pregnancy. *J Mol Endocrinol.* 14:255-261.
3. Maslar IA, Riddick DH. 1979 Prolactin production by human endometrium during the normal menstrual cycle. *Am J Obstet Gynecol.* 14:255-261.



4. Daly DC, Maslar JA, Riddick DH. 1983 Prolactin production during *in vitro* decidualisation of proliferative endometrium. *Am J Obstet Gynecol*. 145:672-675.
5. DiMattia GE, Gellerson B, Duckworth ML, Friesen HG. 1990 Human prolactin gene expression. The use of an alternative noncoding exon in decidua and the IM-9-P3 lymphoblast cell line. *J Biol Chem*. 265:16412-16421.
6. Gellerson B, Kempf R, Telgmann R, DiMattia GE. 1994 Nonpituitary human prolactin gene transcription is independent of Pit-1 and differentially controlled in lymphocytes and in endometrial stroma. *Mol Endocrinol*. 8:356-373.
7. Draca S. 1995 Prolactin as an immunoreactive agent: review. *Immunol Cell Biol*. 73:481-483.
8. Chambers WH, Amosato AA, Smith MS, Kenniston TW, Herberman RB, Appasamy PM. 1995 Prolactin receptor expression by rat NK cells. *Nat Immunol*. 14:145-156.
9. Dardenne M, Do Carmo M, De Moraes L, Kelly PA, Gagnierault M. 1994 Prolactin receptor expression in human haematopoietic tissues analyzed by flow cytometry. *Endocrinology*. 134:2108-2114.
10. Pellegrini I, Lebrun J, Ali S, Kelly PA. 1992 Expression of prolactin and its receptor in human lymphoid cells. *Mol Endocrinol*. 6:1023-1031.
11. Wu H, Devi R, Malarkey WB. 1996 Expression and localization of prolactin messenger ribonucleic acid in the human immune system. *Endocrinology*. 137:349-353.
12. Clevenger CV, Russell DH, Appasamy PM, Prystowsky MB. 1990 Regulation of interleukin 1-driven T-lymphocyte proliferation by prolactin. *Proc Natl Acad Sci USA*. 87:6460-6464.
13. Hartman D, Holaday J, Bernton E. 1989 Inhibition of lymphocyte proliferation by antibodies to prolactin. *FASEB J*. 3:2194-2202.
14. Dineen JK, Kelly JD. 1972 The suppression of rejection of *Nippostrongylus brasiliensis* in lactating rats: the nature of the immunological defect. *Immunology*. 22:1-13.
15. Kelly JD, Dineen JK. 1973 The suppression of rejection of *Nippostrongylus brasiliensis* in Lewis strain rats treated with ovine prolactin. *Immunology*. 24:551-558.
16. Loke YW, King A. 1995 Uterine mucosal leucocytes. In: Loke YW, King A, eds. *Human implantation, cell biology and immunology*. Cambridge: Cambridge University Press; 102-129.
17. Bulmer JN. 1995 Immune cells in decidua. In: Kurpisz M, Fernandez N, eds. *Immunology of human reproduction*. Oxford: BIO Scientific; 313-334.
18. King A, Loke YW. 1991 On the nature and function of human uterine granular lymphocytes. *Immunol Today*. 12:432-435.
19. Healy DL. 1985 Chronic polyhydramnios is a syndrome with a lactogen receptor defect in the chorion laeve. *Br J Obstet Gynaecol*. 92:461-467.
20. Tadokoro N, Koibuchi N, Ohtake H, et al. 1995 Localisation of prolactin and its receptor messenger RNA in the human decidua. *Experientia*. 51:1216-1219.
21. Maaskant RA, Bogie LV, Gilger S, Kelly PA, Bryant-Greenwood GD. 1996 The human prolactin receptor in the fetal membranes, decidua and placenta. *Endocrinology*. 31:396-405.
22. Nevalainen MT, Valve EM, Ingleton PM, Harkonen PL. 1996 Expression and hormone regulation of prolactin receptors in rat dorsal and lateral prostate. *Endocrinology*. 137:3078-3088.
23. Baggiolini M, Dewald B, Moser B. 1997 Human chemokines: an update. *Annu Rev Immunol*. 15:675-705.
24. Loke YW, King A. 1991 Recent developments in the human maternal-fetal immune interaction. *Curr Opin Immunol*. 3:762-766.
25. Clarke LA, Edery M, Loudon ASI, et al. 1995 Expression of the prolactin receptor gene during the breeding and non-breeding seasons in red deer (*Cervus elaphus*): evidence for the expression of two forms in the testis. *J Endocrinol*. 146:313-321.
26. Jikihara H, Kessler CA, Cedars MI, Brar AK. 1996 Up regulation of the human prolactin receptor in the endometrium. *Endocr Rev*. 17:157-162.
27. Wu WX, Glasier A, Norman J, Kelly RW, Baird DT, McNeilly AS. 1990 The effects of the antiprogesterone mifepristone, *in vivo*, and progesterone *in vitro* on prolactin production by the human decidua in early pregnancy. *Hum Reprod*. 5:627-631.
28. Markoff E, Zeitler P, Peleg S, Handwerker S. 1983 Characterisation of the synthesis and release of prolactin by an enriched fraction of human decidua cells. *J Clin Endocrinol Metab*. 56:962-968.
29. Gu Y, Soares MJ, Srivastava RK, Gibor G. 1994 Expression of decidual prolactin-related protein in the rat decidua. *Endocrinology*. 135:1422-1427.
30. Dijane J, Clauser H, Kelly PA. 1979 Rapid down regulation of prolactin receptors in mammary gland and liver. *Biochem Biophys Res Commun*. 90:1371-1375.
31. Muccioli G, Di Carlo R. 1994 Modulation of prolactin receptors in the rat hypothalamus in response to changes in serum concentration of endogenous prolactin or to ovine prolactin administration. *Brain Res*. 663:244-250.
32. Posner BI, Kelly PA, Friesen HG. 1975 Prolactin receptors in rat liver: possible regulation by prolactin. *Science*. 188:57-59.
33. Noyes RW, Hertig AT, Rock J. 1950 Dating the endometrial biopsy. *Fertil Steril*. 1:3-25.
34. Clapp C, Martial JA, Guzman RC, Rentier-Delrue F, Weiner RI. 1993 The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. *Endocrinology*. 133:1292-1299.
35. Clapp C, Weiner RI. 1992 A specific, high affinity, saturable binding site for the 16-kilodalton fragment of prolactin on capillary endothelial cells. *Endocrinology*. 130:1380-1386.
36. Inoue T, Kanzaki H, Imai K, et al. 1996 Progesterone stimulates the induction of human endometrial CD56<sup>+</sup> lymphocytes in an *in vitro* culture system. *J Clin Endocrinol Metab*. 81:1502-1507.
37. King A, Gardner L, Loke YW. 1996 Evaluation of oestrogen and progesterone receptor expression in uterine mucosal lymphocytes. *Hum Reprod*. 11:1079-1082.
38. Jones RL, Kelly RW, Critchley HOD. 1997 Chemokine expression in human endometrium coincides with leukocyte accumulation. *Hum Reprod*. 12:1300-1306.
39. Bowen JM, Landis Keyes P, Warren JS, Townson DH. 1996 Prolactin-induced regression of the rat corpus luteum: expression of monocyte chemoattractant protein-1 and invasion of macrophages. *Biol Reprod*. 54:1120-1127.
40. Koay ESC, Bryant-Greenwood GD, Yamamoto SY, Greenwood FC. 1986 The human fetal membranes: a target tissue for relaxin. *J Clin Endocrinol Metab*. 62:513-521.

# Morphological and functional features of endometrial decidualization following long-term intrauterine levonorgestrel delivery

R07076

AUTHOR'S COPY

H.O.D.Critchley<sup>1,4</sup>, H.Wang<sup>1</sup>, R.Jones<sup>1</sup>, R.W.Kelly<sup>2</sup>, T.Drudy<sup>1</sup>, A.Gebbie<sup>1</sup>, C.H.Buckley<sup>2</sup>, A.S.McNeilly<sup>2</sup> and A.F.Glasier<sup>1</sup>

<sup>1</sup>Department of Obstetrics and Gynaecology, <sup>2</sup>MRC Reproductive Biology Unit, University of Edinburgh, Centre for Reproductive Biology, Edinburgh EH3 9EW and <sup>3</sup>Department of Reproductive Pathology, University of Manchester, Manchester, UK

<sup>4</sup>To whom correspondence should be addressed

Irregular bleeding remains a common reason for the discontinuation of progestin-only contraception. The levonorgestrel releasing intrauterine system (LNG-IUS) has profound morphological effects upon the endometrium. Specific features are gland atrophy and extensive decidual transformation of the stroma. Morphological changes in the endometrium may be associated with perturbation of mechanisms regulating normal endometrial function. This study describes endometrial stromal and glandular features prior to and up to 12 months following insertion of the LNG-IUS. Comparison is made with first trimester decidua. In order to elucidate further mechanisms governing endometrial function with local intrauterine delivery of LNG, we here report histological features consistent with decidualization; a significant increase in granulocyte-macrophage colony stimulating factor (GM-CSF) immunoreactivity in decidualized stromal cells; glandular and stromal prolactin receptor expression and an infiltrate of CD56+ large granular lymphocytes and CD68+ macrophages. We are unaware of previous reports which have documented longitudinally both morphological and functional observations in endometrium exposed to local intrauterine levonorgestrel delivery. These studies demonstrate that long-term administration of intrauterine levonorgestrel results in features of altered morphology and function. No correlation was apparent between the end points in the study and the bleeding patterns described by the subjects. Further evaluation of these features in the context of menstrual bleeding experience may contribute to a better understanding of this troublesome side-effect which often leads to dissatisfaction and discontinuation of the intrauterine system.

**Key words:** decidualization/GM-CSF/intrauterine levonorgestrel/prolactin receptor/leukocytes

## Introduction

Irregular bleeding remains the single most common indication for discontinuation of progesterone-only methods of contracep-

tion (Findlay, 1996) and the aetiology of this frequent complaint is not understood. Few data exist concerning endometrial stromal changes in users of progestin only contraception. Indeed attention has been drawn to the fact that the 'short phase of decidualization' observed following commencement of progesterone only contraception may be transient and thus the cells are not 'true decidual cells' with the consequence that factors not normally associated with decidual cells may be produced (Findlay, 1996).

Steroid releasing intrauterine devices have profound morphological effects on the endometrium. Specific features are suppression of proliferative activity and atrophy with extensive decidual transformation of the stroma (Silverberg *et al.*, 1986; Sheppard, 1987). Intrauterine devices elicit characteristic local changes in leukocyte populations within the endometrium, including infiltration of macrophages, lymphocytes and neutrophils which appear in the stroma and epithelium (Sheppard, 1987). Mechanisms concerned with leukocyte distribution in the endometrium of IUD users are not known. During the normal menstrual cycle there is a late secretory phase increase in stromal leukocytes (Bulmer *et al.*, 1988) with a pre-menstrual increase in stromal macrophages (Kamat and Isaacson, 1987). Both the late secretory phase endometrium and early decidua contain a significant population of leukocytes, the majority of which are CD56+ large granular lymphocytes (LGL) (King and Loke, 1991).

The temporal expression of prolactin in the uterus implicates a role for this hormone in decidualization and hence implantation (Wu *et al.*, 1995). Prolactin is an immunoregulating agent and may play a role in leukocyte function. Our group (Jones *et al.*, 1998) has recently reported the localization of the prolactin receptor protein in glandular epithelium and some stromal cells from the mid to late secretory phase of the menstrual cycle and in first trimester decidua, coinciding with the onset of decidualization.

Few data are available concerning endometrial cytokines and their role in mechanisms concerned with endometrial bleeding. Granulocyte-macrophage colony stimulating factor (GM-CSF) is an activating growth factor for granulocytic and monocytic cells. Human GM-CSF is considered to be steroid-regulated (Sharpe-Timms *et al.*, 1994). GM-CSF is synthesized by epithelial cells in the murine pregnant and non-pregnant uterus (Robertson *et al.*, 1992) and is likely to be regulated in the mouse primarily by oestrogen. In the human, epithelial cells are the major contributor to production (Giacomini *et al.*, 1995) and variations are reported in the level of immunohistochemical expression of GM-CSF in endometrial epithelial cells (Sharpe-Timms *et al.*, 1994). Furthermore, GM-CSF production by resident leukocytes (macrophage and

large granular lymphocytes) has been demonstrated (Jokhi *et al.*, 1994). A potential role for endometrial GM-CSF in macrophage recruitment into the stroma has been reported in rodents (Robertson and Seamark, 1992).

This study describes endometrial stromal and glandular features prior to and up to 12 months following insertion of a levonorgestrel-releasing intrauterine system (LNG-IUS; Mirena, Leiras Oy, Finland). The LNG-IUS has an inert frame on which a silastic capsule has been attached to the vertical arm, releasing 20 µg levonorgestrel every 24 h to the uterine cavity from a total load of 52 mg LNG (Luukkainen *et al.*, 1990). A comparison has been made with the morphological features of first trimester decidua.

## Materials and methods

Ethical approval for the study was obtained from Lothian Research Ethics Committee (reference: 1702/94/6/44). Informed consent was obtained from 14 women aged between 32 and 48 years (median age is 37 years). All subjects were fertile, described regular menstrual cycles (cycle length 25–35 days) and were not using hormonal or intrauterine contraception in the 6 months prior to inclusion in the study. The indication for insertion of the LNG-IUS was either for contraception ( $n = 10$ ) or heavy menstruation ( $n = 4$ ). The study was longitudinal with each subject acting as her own control. All subjects underwent a pre-insertion endometrial biopsy either in the proliferative ( $n = 7$ ) or secretory ( $n = 7$ ) phase of the cycle. The stage of the cycle prior to LNG-IUS insertion was defined according to the criteria of Noyes *et al.* (1950). Biopsies were performed in an outpatient setting with a pipelle suction curette (Laboratoire CCD, Paris, France). Further endometrial samples were collected 1, 3, 6 and 12 months following insertion of the LNG-IUS. Once the LNG-IUS was *in situ*, the histological appearance of endometrium samples was indistinguishable whether collected in the follicular or luteal phase, hence data at time period of 1, 3, 6 and 12 months were pooled.

In addition decidua tissue was collected from ten women (8–10 weeks amenorrhoea) undergoing surgical termination of pregnancy. Decidual biopsies were stained with a monoclonal antibody against cytokeratin to confirm absence of trophoblast tissue (decidua parietalis). All endometrial and decidua tissue samples were fixed overnight in 10% neutral buffered formalin at 4°C, rinsed and stored in 70% ethanol and thereafter routinely wax embedded. Five micron sections were cut for routine histopathology (haematoxylin and eosin staining) and immunohistochemical localization of granulocyte macrophage colony stimulating factor (GM-CSF); prolactin receptor, CD56+ large granular lymphocyte (LGL), macrophage (CD68) and neutrophil elastase immunoreactivity. Our laboratory has recently described the localization of progesterone receptors (subtypes A + B) and oestrogen receptors in normal endometrium and decidua (Wang *et al.*, 1998) and in endometrium exposed to intrauterine LNG (Critchley *et al.*, 1998).

## Immunohistochemistry procedures

### Prolactin receptor immunolocalization

Paraffin sections were dewaxed and rehydrated through descending grades of ethanol to distilled water. The sections were then washed twice in 0.1 M phosphate buffered saline (PBS) for 5 min. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide ( $H_2O_2$ , BDH Laboratory Supplies, Poole, UK) in distilled water for 5 min and then sections were again washed. Non-specific binding was reduced by a 20 min incubation with non-immune goat serum (Vector Stain Elite<sup>®</sup>; Vector Laboratories, Peterborough, UK)

in a humidified chamber at room temperature, following which the excess was carefully removed and the primary antibody (Nevalainen *et al.*, 1996) (rabbit polyclonal raised against a rat peptide sequence common to the short and long form of the receptor, kindly supplied by Dr P. Ingleton, University of Sheffield) was applied at a dilution of 1:50. Slides were incubated overnight ( $17 \pm 1$  h) at 4°C. Antibody binding was detected by the sequential application of biotinylated goat anti-rabbit IgG and an avidin biotin peroxidase complex (ABC Vector Stain<sup>®</sup>; Vector Laboratories).

The substrate 3,3'-diaminobenzidine (DAB, Vector Laboratories) was then utilized to visualize positive immunoreactivity. Finally, sections were counter-stained with Harris' haematoxylin (Pioneer Research Chemicals Ltd, Colchester, UK), dehydrated and cleared in xylene before mounting in Pertex<sup>®</sup> (Cellpath, Hemel Hempstead, UK).

Positive controls included for the prolactin receptor immunohistochemistry were sheep pituitary sections (Tortones *et al.*, 1996) and human term fetal membranes (Maaskant *et al.*, 1996). Negative controls had an equivalent concentration of non-immune rabbit immunoglobulin substituted for the primary antibody to exclude the possibility of non-specific binding.

### Granulocyte macrophage colony stimulating factor (GM-CSF)

#### Immunolocalization

A similar protocol as above was used for the localization of GM-CSF. The primary antibody was raised in the mouse against human GM-CSF [GM-CSF (ZM-213), Genzyme Diagnostics, Kent, UK]. Incubation with the primary monoclonal antibody at a dilution of 1:75 took place for 90 min at 37°C. Thereafter the protocol was similar to that employed for prolactin receptor. Tris buffered saline (TBS) was used throughout instead of PBS. The second antibody was a horse anti-mouse antibody (Vector Laboratories) and the avidin biotin peroxidase complex ABC Elite<sup>®</sup> (Vector Laboratories). Negative controls were performed by replacing the primary antibody with mouse immunoglobulin at the same dilution (1:75).

The positive controls were a GM-CSF expressing cell line (MG-63 cells, derived from a male osteosarcoma cell line and gift of Dr Fouad Habib, Western General Hospital, Edinburgh). MG-63 cells were grown up on Chamber slides (Nunc Inc, Naperville, IL, USA). After fixation, the MG-63 slides were stored in 70% ethanol until use. Cells were first washed separately in TBS for 5 min and then included as a positive control.

#### Leukocyte immunolocalization

Sections (5 µm) from formalin-fixed, paraffin embedded biopsies were cut for immunohistochemical localization of (i) macrophages (CD68, DAKO, Code M876); (ii) CD56+ lymphocytes (Zymed, San Francisco, CA, USA); (iii) neutrophil elastase (DAKO Neutrophil Elastase, Code M752). Tissue sections were dewaxed and rehydrated. Non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in distilled water for 5 min at room temperature. Pre-treatment of the sections was necessary for localization of macrophages and CD56+ lymphocytes as described below.

For macrophage (CD 68) immunostaining, tissue sections were exposed to an enzyme digestion with 0.1% trypsin in 0.1% calcium chloride at pH 7.8. The digestion was conducted at 37°C for 25 min and subsequent enzyme activity removed by washing in tap water.

For CD56+ lymphocyte immunostaining, tissue sections were microwaved at high power in 0.01 M sodium citrate buffer (pH 6.0) for 20 min then allowed to stand for a further 20 min. Sections were washed in buffer prior to a non-immune block.

Thereafter all sections were exposed to a non-immune block with normal horse serum for 20 min at room temperature. Tissue sections were subsequently incubated with the appropriate primary antibody:





Figure 1. Photomicrograph of endometrium exposed to intrauterine levonorgestrel for 3 months. The glands are narrow and inactive and the stroma is typically decidualized. There is a scanty granulated lymphocyte infiltrate. Original magnification  $\times 185$ .

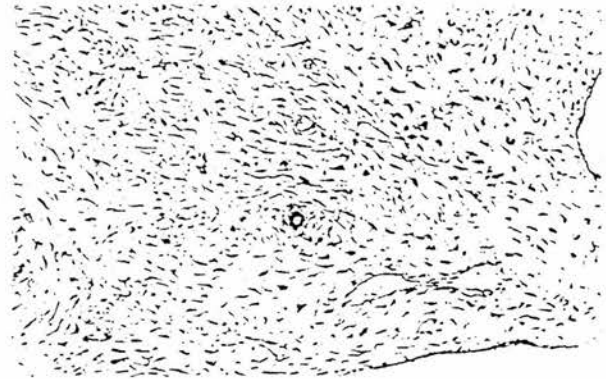


Figure 2. An example of the spindle-celled pattern of pseudo-decidualization of the stroma. The central gland is very narrow and inactive. Levonorgestrel intrauterine system *in situ* for 12 months. Original magnification  $\times 185$ .

- (i) macrophage antibody (CD68) dilution 1:50 for 60 min at 37°C;
- (ii) CD56 antibody dilution 1:250 for 60 min at 37°C;
- (iii) neutrophil elastase antibody dilution 1:50 for 60 min at 37°C.

Sections were labelled with an avidin-biotin-peroxidase detection system (Vector Stain<sup>®</sup>; Vector Laboratories). Colour development employed diaminobenzidine (DAB) solution for 2–10 min. Subsequently tissue sections were counterstained with haematoxylin, dehydrated and cleared in xylene and mounted in Pertex. Negative controls were conducted by replacing the primary antibody with non-immunized mouse immunoglobulin at the same concentration as the primary antibody.

Immunostaining intensity and distribution of epitopes in all tissue sections were assessed semi-quantitatively, on a four point scale: 0 = no staining, 1 = faint staining, 2 = moderate staining, 3 = intense staining. Scoring was performed blind by two observers. The mean and standard error of mean ( $\pm$ SEM) was calculated. The data were analysed by one-way analysis of variance (ANOVA), using Fisher's PLSD coefficient to assign significance.

## Results

### Histology

The LNG-IUS produced widespread morphological changes in the endometrium (Figures 1 and 2). The histological changes were not limited to the contact site although typical contact site changes were observed in the majority of cases. The superficial part of the endometrium formed cushions of rather oedematous, spindle-celled, pseudo-decidualized stroma (Figure 2) and occasionally, microscopic, oedematous, surface micropapillae were seen. In biopsies where the progestational effect was long-standing, the endometrial decidualization remote from the device was spindle-celled and diminished in intensity whilst the decidualization nearest to the device remained more typical with 'rounded' cells rather than spindle cells. Glands in the superficial part of the endometrium were extremely narrow, whilst those in the deeper layers were slightly wider and lined by an epithelium which was cubo-columnar in contrast to the cells lining the superficial glands which were flattened cuboidal. In a small number of biopsies

haemorrhagic infarction, necrobiosis or coagulative necrosis was present.

### Immunohistochemistry

#### GM-CSF immunostaining

Positive immunostaining (brown) for GM-CSF was observed in the cytoplasm of glandular and surface epithelial cells in normal endometrium. In early decidua (Figures 3A and 4) positive immunoreactivity in epithelial and decidualized stromal cells, especially in a perivascular location, was noted. Immunoreactivity tended to be heterogeneous. Regions where decidualization was marked demonstrated strongest immunoreactivity. Strong cytoplasmic glandular and stromal immunoreactivity, particularly in a perivascular location, was also evident following insertion of the LNG-IUS (Figures 3B and 4). There was a significant increase ( $P < 0.05$ ) in GM-CSF immunoreactivity in the stromal compartment of LNG-exposed endometrium at 1, 3, 6 and 12 months post insertion compared with 'pre-insertion' endometrium biopsied in both the proliferative ( $P < 0.01$ ) and secretory phase ( $P < 0.05$ ) of the cycle (Figure 4). Immunoreactivity in normal decidual stromal cells was similar to secretory phase endometrium (see Figure 4B, lower panel).

#### Prolactin-receptor immunostaining

Minimal positive immunostaining was observed in pre-insertion endometrial biopsies collected in the proliferative phase. However, in pre-insertion endometrial biopsies from the secretory phase, positive immunoreactivity was evident in the cytoplasm of glandular epithelium and in some stromal cells. Biopsies of first trimester decidua displayed strong immunoreactivity in glands and decidualized stromal cells (Figure 3C). Consistent with these features of prolactin receptor immunoreactivity in early decidual tissue, biopsies collected post insertion of the LNG-IUS displayed positive immunostaining both in the glandular epithelium and in pseudo-decidualized stromal cells (Figure 3D). A significant increase ( $P < 0.05$ ) in stromal prolactin receptor immunostaining

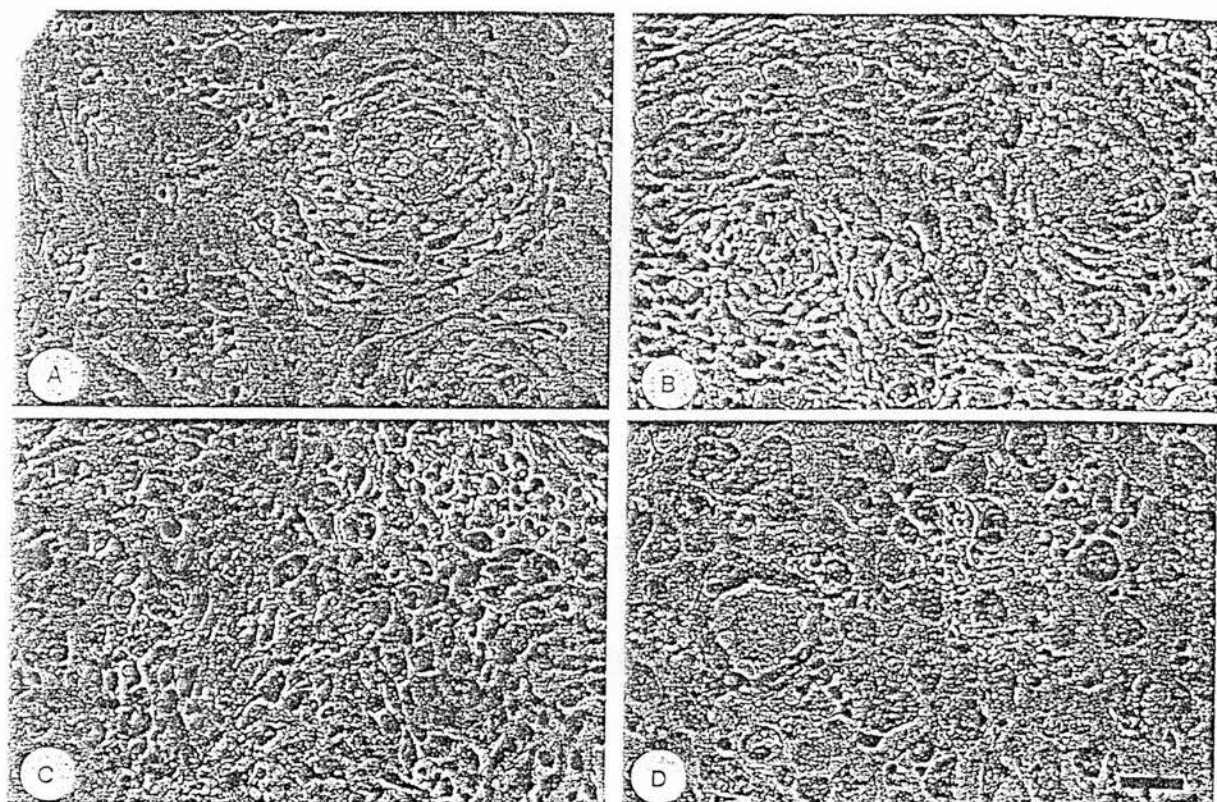


Figure 3. Photomicrographs of granulocyte-macrophage colony stimulating factor (GM-CSF) immunostaining in: A. First trimester decidua. Positive immunoreactivity (brown staining) is noted particularly in the cytoplasm of large decidualized cells often in a perivascular location. B. Endometrial biopsy from patient 1 month after insertion of levonorgestrel releasing intrauterine system (LNG-IUS). Strong cytoplasmic immunoreactivity is evident in the stroma. Photomicrographs of prolactin receptor immunostaining in: C. First trimester decidua. Positive immunoreactivity (brown) is clearly visible in decidualized stromal cells. D. Endometrial biopsy collected 3 months post LNG-IUS insertion displaying positive immunostaining in both glandular epithelium and decidualized stroma. Scale bar = 25  $\mu$ m.

was evident following insertion of the LNG-IUS (Figure 5, lower panel).

#### Leukocyte immunostaining

Intense positive immunostaining of CD56+ LGL was evident in all endometrial biopsies collected following insertion of the intrauterine system. There was significantly greater ( $P < 0.05$ ) CD56+ immunoreactivity in the stroma of secretory compared to proliferative endometrium, prior to insertion of the LNG-IUS (Figure 6B). The significant increase ( $P < 0.05$ ) was still evident at 1 and 3 months post insertion of the LNG-IUS when compared to proliferative endometrium prior to LNG-IUS insertion (Figure 6B). Strong CD56+ immunostaining was observed however in normal decidua, especially in areas of full decidualization. Positive immunoreactivity for CD68+ macrophages was also obtained in all endometrial biopsies following insertion of the LNG-IUS (Figure 6A). There was a significant increase in macrophage immunostaining between proliferative endometrium pre-insertion and the 1st month post insertion ( $P < 0.05$ ). A significant decrease ( $P < 0.05$ ) in CD68+ macrophage immunoreactivity was noted from 1–12 months post insertion. There was negligible immunostaining for neutrophil elastase (polymorphonuclear leukocyte marker) in all post insertion endometrial biopsies (data not shown).

#### Discussion

In order to elucidate further local mechanisms governing endometrial function with local intrauterine delivery of levonorgestrel, the present study has reported histological features consistent with decidualization. We have observed a significant increase in GM-CSF immunoreactivity, particularly in the decidualized stromal cells observed in LNG exposed endometrium, plus glandular and stromal prolactin receptor expression and an infiltrate of CD56+ large granular lymphocytes and CD68+ macrophages. We are unaware of any previous reports which have documented longitudinally both morphological and functional observations in endometrium exposed to local intrauterine LNG delivery.

The endometrial morphological features following intrauterine levonorgestrel delivery are typical of those seen in long-term users of a progestogen (Silverberg *et al.*, 1986; Buckley and Fox, 1989). The features of pseudo-decidualization closely resembled the morphology of early pregnancy decidua.

The progestational effects observed in this study occurred throughout the endometrium and are consistent with an earlier report (Nilsson *et al.*, 1978), and were not limited to the immediate vicinity of the device. The endometrial biopsies

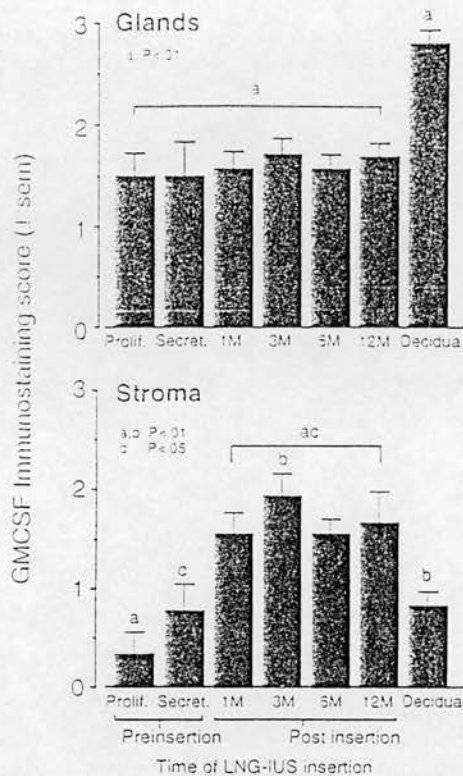


Figure 4. Granulocyte-macrophage colony stimulating factor (GM-CSF) immunostaining scores (mean  $\pm$  SEM) in endometrial glands (upper panel) and stroma (lower panel) prior to and following insertion of levonorgestrel releasing intrauterine system (LNG-IUS). Prolif = proliferative phase, Secret = secretory phase, M = months.

examined in the present study did not display the micropolyps described by Silverberg *et al.* (1986) where the median duration of use was 48 months and women using a device releasing 20  $\mu$ g daily were all examined after at least 12 months of use. The lack of observation of micropolyps in our study may be due to the fact that the levonorgestrel intrauterine system had only been *in situ* for up to 12 months.

In a few biopsies haemorrhagic infarction, necrobiosis and coagulative necrosis were present. These features are indicative of spontaneous tissue breakdown but differ from that seen in normal menstruation, in that the features were usually focal or multifocal. All subjects in this study had recorded details of daily bleeding (if present) for the full 12 months study period. On review, the biopsies with evidence of spontaneous tissue breakdown were not from subjects who had described break-through bleeding (unpublished data).

Any agent which modifies endometrial morphology is likely also to modify the normal intrauterine processes regulating function. The stromal GM-CSF immunoreactivity in the local levonorgestrel treated endometrium closely resembled GM-CSF immunostaining in the epithelial and stromal cells of first trimester decidua. Particularly of note was the localization of immunostaining to large perivascular decidualized cells. Appreciable amounts of GM-CSF are known to be produced by first trimester decidua (Jokhi *et al.*, 1994) and a potential

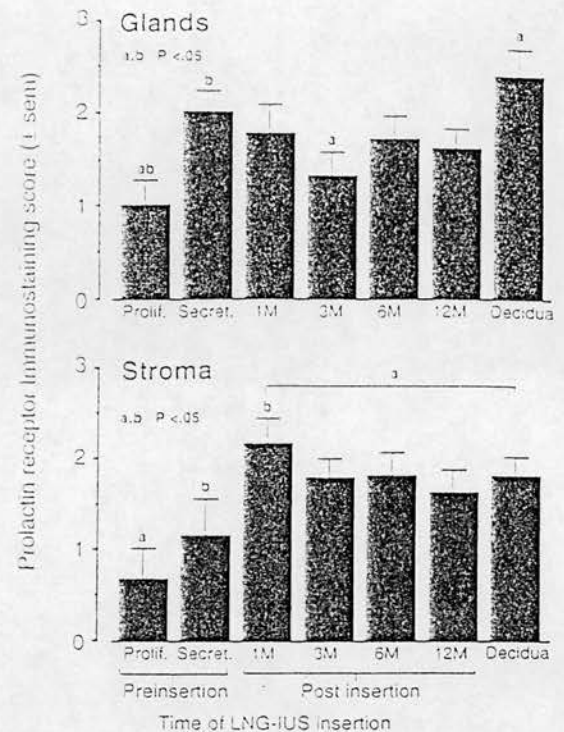


Figure 5. Prolactin receptor immunostaining scores (mean  $\pm$  SEM) in endometrial glands (upper panel) and stroma (lower panel) prior to and post insertion of a levonorgestrel releasing intrauterine system (LNG-IUS).

role in leukocyte recruitment has been proposed (Robertson and Seamark, 1992).

Prolactin receptor protein has been immunolocalized to the glandular epithelium and stromal cells of pseudo-decidualized endometrium following intrauterine levonorgestrel delivery. Our observation is consistent with data from our group (Jones *et al.*, 1998) concerning expression of the prolactin receptor in both normal endometrium and first trimester decidua. Locally derived growth factors and cytokines are likely to regulate prolactin receptor expression (Gu *et al.*, 1994), and the detection of strong immunoreactivity for the receptor in both epithelium and stroma in pseudo-decidualized endometrium suggests a paracrine role for prolactin.

The pseudo-decidualized endometrium associated with local levonorgestrel delivery displayed a significant increase in CD56+ LGL when compared with proliferative phase endometrium and has a persistent population of macrophages. Normal decidua has a similar leukocyte population (Loke and King, 1995). The mode of recruitment of these cell types remains under investigation, although, under the influence of progesterone, neither cell type to date has been reported to express sex steroid receptors (King *et al.*, 1996). Local intrauterine levonorgestrel delivery results in a down-regulation of stromal progesterone receptors (Critchley *et al.*, 1998) at a time where, in the current study, we have observed strong prolactin receptor immunoreactivity that increased significantly 1 month post insertion of the LNG-IUS.

From the above data it must be concluded that the stromal



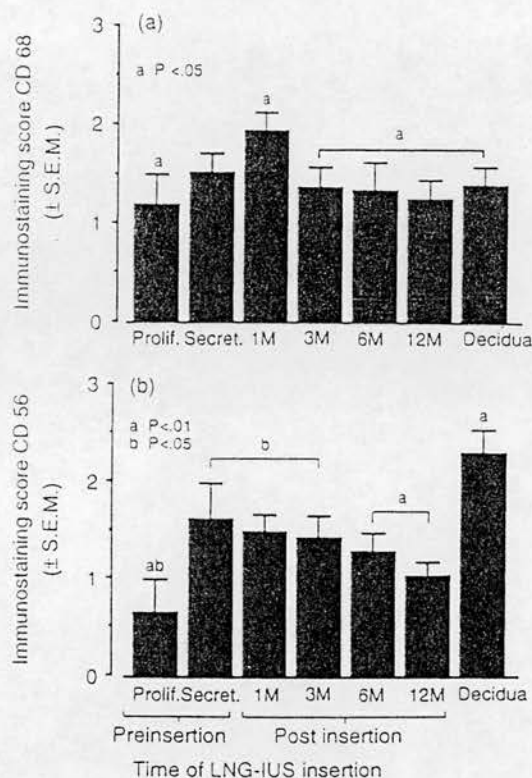


Figure 6. Mean ( $\pm$  SEM) immunostaining scores for (A) macrophages (CD68+) and (B) large granular lymphocytes (CD56+) in endometrial stroma before and after exposure to intrauterine levonorgestrel.

compartment of the endometrium does indeed undergo changes consistent with decidualization for at least up to 12 months post insertion of LNG-IUS. Previous examination of the state of the endometrial stroma in progestin-only contraceptive users was limited (Findlay, 1996). Furthermore, it has been suggested (Findlay, 1996) that there may be a short phase of decidualization following commencement of the method. In this study evidence is presented that GM-CSF and prolactin receptor proteins immunolocalize to the pseudo-decidualized cells in a similar manner to the true decidual cells of first trimester pregnancy. It is notable that GM-CSF protein localized particularly to large decidualized perivascular cells. Findlay (1996) has noted that in progestin exposed endometrium the decidualized cells may produce factors not normally associated with true decidualized cells which may have the potential to be detrimental to blood vessels.

It is worth noting that in normal decidua immunoreactivity was heterogeneous, and was most marked for GM-CSF, prolactin receptor and CD56 where decidualization was apparent. In these early pregnancy biopsies some regions still displayed a marked glandular morphology. It is recognised that during the first trimester, two histologically distinguishable zones may be observed (Bell, 1991).

The presence of a marked and persistent population of CD56+ LGL is also consistent with the observation that uterine LGL are the predominant lymphocyte reported in first

trimester decidua (Loke and King, 1997). Furthermore, in this study macrophages were evident throughout the 12 months following insertion of a LNG-IUS. In this context Clark *et al.* (1996) reported a reduced frequency of CD68+ cells in the atrophic endometrium of Norplant® users (sub-dermal levonorgestrel). The number of CD68+ cells was significantly increased in those women using Norplant with abnormal bleeding. In the context of the present study there was a significant increase in macrophage immunoreactivity at 1 month post LNG-IUS insertion and proliferative phase endometrium. Interestingly, thereafter a significant decrease in macrophage immunostaining was observed between 1 and 12 month following insertion of LNG-IUS. No formal quantification of immunostaining was undertaken due to the small size of many of the later biopsies, and hence the description was subjective.

Bleeding disorders remain a major problem and a primary reason for discontinuation of progestin-only methods of contraception (Findlay, 1996). A previous irregular menstrual history has been raised as a potential contributing factor to bleeding disorders. In the present study all women recruited described a regular bleeding history. Many of them went on to experience irregular bleeding with the LNG-IUS *in situ*. A minority ( $n = 4$ ) also described heavy loss. Nine out of 14 women (64%) experienced light menstrual blood loss each month and only one subject achieved amenorrhoea with the LNG-IUS (unpublished data). Despite detailed study of the several end points in this study, no correlation was established with the menstrual bleeding patterns described by the subjects.

A detailed understanding of the local endometrial mechanisms which are disturbed by the presence of high dose intrauterine progestagen are also important with respect to the contraceptive action of the LNG-IUS. Recently Mandelin *et al.* (1997) described inappropriate glycodeclin A mRNA and protein expression before and at mid cycle in women using a LNG-IUS. This untimely production of glycodeclin A has been implicated in the contraceptive action of the LNG-IUS. In this context glycodeclin A is usually absent from the endometrium in the proliferative and immediate postovulatory phases, i.e. at a time when glandular progesterone receptor expression is maximal. It is interesting to note that the inappropriate production of glycodeclin A coincides with the down regulation of progesterone receptor reported (Critchley *et al.*, 1998) with intrauterine levonorgestrel administration.

In summary, the present study has described features consistent with endometrial decidualization in the presence of an intrauterine levonorgestrel-releasing system. It is not possible to determine whether these decidualized cells are 'true decidual cells' (Findlay, 1996) although several of the features described herein are almost indistinguishable from normal decidua. Further study of the decidualized nature of the stromal cells in the levonorgestrel exposed endometrium should contribute to a greater understanding of the mechanisms responsible for break-through bleeding, a major reason for discontent with progestin-only contraception.

#### Acknowledgements

We wish to acknowledge the assistance of Dr Sharon Cameron with endometrial biopsy collection. We are also grateful to Professor

Robert M. Brenner for his helpful comments during preparation of the manuscript, Mr Tom McFetters and Mr Ted Pinner for help with the illustrations and to Mrs Vicky Watters for secretarial assistance. The Levonorgestrel intrauterine systems were provided by Leiras Oy, Finland. This study was supported by Wellcome Trust Project Grant No: 044744/Z/95/Z.

## References

- Bell, S.C. (1991) The insulin-like growth factor binding proteins the endometrium and decidua. *Ann. N. Y. Acad. Sci.*, **622**, 120-137.
- Buckley, C.H. and Fox, H. (1989) The effect of therapeutic and contraceptive hormones on the endometrium. In Gottlieb, L.S., Neville, A.M. and Walker, F. (eds) *Biopsy pathology of the endometrium*. Biopsy Pathology Series, vol. 14. Chapman and Hall, London, pp. 68-92.
- Bulmer, J.N., Lunny, D.P. and Hagin, S.V. (1988) Immunohistochemical characterisation of stromal leucocyte in non-pregnant human endometrium. *Am. J. Reprod. Immunol.*, **17**, 83-90.
- Clark, D.A., Wang, S., Rogers, P. et al. (1996) Endometrial lymphomyeloid cells in abnormal uterine bleeding due to levonorgestrel (Norplant). *Hum. Reprod.*, **11**, 1438-1444.
- Critchley, H.O.D., Kelly, R.W., Lea, R.G. et al. (1996) Sex steroid regulation of leucocyte traffic in human decidua. *Hum. Reprod.*, **11**, 2257-2262.
- Critchley, H.O.D., Wang, H., Kelly, R.W. et al. (1998) Progesterone receptor isoforms and prostaglandin dehydrogenase in the endometrium of women using a levonorgestrel-releasing intrauterine system. *Hum. Reprod.* (in press).
- Findlay, J.K. (1996) Future direction for research on endometrial bleeding. *Hum. Reprod.*, **11** (suppl.2), 179-183.
- Giacomini, G., Tabibzadeh, S.S., Satyaswaroop, P.G. et al. (1995) Epithelial cells are the major source of biologically active granulocyte-macrophage colony-stimulating factor in human endometrium. *Hum. Reprod.*, **10**, 3259-3263.
- Gu, Y., Soares, M.J., Srivastava, R.K. and Gibor, G. (1994) Expression of decidual prolactin-related protein in the rat decidua. *Endocrinology*, **135**, 1422-1427.
- Jokhi, P.P., King, A. and Loke, Y.W. (1994) Production of granulocyte-macrophage colony-stimulating factor by human trophoblast cells and by decidual large granular lymphocytes. *Hum. Reprod.*, **9**, 1660-1669.
- Jones, R.L., Critchley, H.O.D., Brooks, J. et al. (1998) Localisation and temporal expression of prolactin receptor in human endometrium. *J. Clin. Endocrinol. Metabol.* (in press).
- Kamat, B.R. and Isaacson, P.G. (1987) The immunocytochemical distribution of leucocyte subpopulations in human endometrium. *Am. J. Pathol.*, **127**, 66-73.
- King, A., Gardner, L. and Loke, Y.W. (1996) Evaluation of oestrogen and progesterone receptor expression in uterine mucosal lymphocytes. *Hum. Reprod.*, **11**, 1079-1082.
- King, A. and Loke, Y.W. (1991) On the nature and function of human uterine granular lymphocytes. *Immunology Today*, **12**, 432-435.
- Loke, Y.W. and King, A. (1995) Uterine mucosal leukocytes. In Loke, Y.W. and King, A. (eds) *Human implantation. Cell biology and immunology*. Cambridge University Press, Cambridge, pp. 102-129.
- Loke, Y.W. and King, A. (1997) Immunology of human placental implantation: clinical implications of our current understanding. *Mol. Med. Today*, **3**, 153-159.
- Luukkainen, T., Lahteenmaki, P. and Toivonen, J. (1990) Levonorgestrel-releasing intrauterine device. *Ann. Med.*, **22**, 85-90.
- Mandelin, E., Koistinen, H., Koistinen, R. et al. (1997) Levonorgestrel-releasing intrauterine device-wearing women express contraceptive glycodefin A in endometrium during midcycle: another contraceptive mechanism? *Hum. Reprod.*, **12**, 2671-2675.
- Maaskant, R.A., Bogie, L.V., Gilger, S. et al. (1996) The human prolactin receptor in the fetal membranes, decidua and placenta. *Endocrinology*, **81**, 396-405.
- Nevalainen, M.T., Valve, E.M., Ingleton, P.M. and Harkonen, P.L. (1996) Expression and hormone regulation of prolactin receptors in rat dorsal and lateral prostate. *Endocrinology*, **137**, 3078-3088.
- Nilsson, C.G., Luukkainen, T. and Arko, H. (1978) Endometrial morphology of women using a D-norgestrel-releasing intrauterine device. *Fertil. Steril.*, **29**, 397-401.
- Noyes, R.W., Hertig, D.T. and Rock, J. (1950) Dating the endometrial biopsy. *Fertil. Steril.*, **1**, 3-25.
- Robertson, S.A., Mayrhofer, G. and Seamark, R.F. (1992) Uterine epithelial cells synthesize granulocyte macrophage colony-stimulating factor and interleukin-6 in pregnant and non pregnant mice. *Biol. Reprod.*, **46**, 1069-1079.
- Robertson, S.A. and Seamark, R.F. (1992) Granulocyte macrophage colony stimulating factor (Gm-CSF): one of a family of epithelial cell-derived cytokines in the pre-implantation uterus. *Reprod. Fertil. Dev.*, **4**, 435-443.
- Sharpe-Timms, K.L., Bruno, P.L., Penney, L.L. and Bickel, J.T. (1994) Immunohistochemical localisation of granulocyte-macrophage colony stimulating factor in matched endometriosis and endometrial tissue. *Am. J. Obstet. Gynecol.*, **17**, 740-745.
- Sheppard, B.L. (1987) Endometrial morphological changes in IUD users: a review. *Contraception*, **36**, 1-10.
- Silverberg, S.G., Haukkamaa, M., Arko, H. et al. (1986) Endometrial morphology during long-term use of levonorgestrel-releasing intrauterine devices. *Int. J. Gynecol. Pathol.*, **5**, 235-241.
- Tortorella, D.J., Brooks, J., Ingleton, P. et al. (1996) Prolactin receptor gene expression and specific translation of the signal in the ovine anterior hypothalamus and pituitary gonadotrophs. 10th International Congress of Endocrinology, p. 1673.
- Wang, H., Critchley, H.O.D., Kelly, R.W. et al. (1998) Progesterone receptor subtype B is differentially regulated in human endometrial stroma. *Mol. Hum. Reprod.* (in press).
- Wu, W.-X., Brooks, J., Glasier, A.F. and McNeilly, A.S. (1995) The relationship between decidualisation and prolactin mRNA and production at different stages of human pregnancy. *J. Mol. Endocrinol.*, **14**, 255-261.

Received on November 17, 1997; accepted on February 13, 1998